

Studies on altered gene expression in  
*Theileria annulata* infected cells and in uninfected  
cells of a related lineage

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## Abstract

The macroschizont stage of the protozoan parasite *Theileria annulata* has the unique ability to immortalise the bovine leukocyte in which it resides. Little is known of the mechanisms by which the parasite is able to induce immortalisation, but a number of associated changes to host cell gene expression have been documented. One such modification is an alteration in the profile of surface polypeptides expressed by the infected cell. One of these infection associated surface antigens is recognised by monoclonal antibody 4H5. In *T. annulata* infected cells this molecule was shown to have a variable molecular mass of between 90 and 125 kDa when different cell lines were analysed. Immune bovine sera failed to recognise this antigen and it is likely that it is encoded by a bovine gene, the expression of which is up-regulated by the presence of the parasite. Characterisation of this antigen and investigation of how its production is elevated upon infection would provide insights into its possible function, and allow a clearer understanding of how the parasite modulates host cell gene expression. To initiate these studies immunoaffinity chromatography was used to purify the antigen (TaHBL20/125) from *T. annulata* (Hissar) infected cells, and the N terminus of the polypeptide was sequenced. Homology was found between this peptide sequence and the human intracellular adhesion molecule, ICAM-1. Information from the amino acid sequence data was then used to design degenerate oligonucleotide pools and an infected cell cDNA library was screened with these probes. Considerable problems were encountered in the design of the oligonucleotides and the gene of interest was not isolated. However, establishment of antigen purification protocols provide the basis for further study.

*T. annulata* is known to infect cells of the monocyte/macrophage and B cell lineages. TaHBL20/125 could therefore represent an up-regulated host molecule associated with cells of a related lineage. Monoclonal antibody 4H5 was found to recognise an antigen of 160 kDa in the human leukemic cell line HL-60. Furthermore, differentiation of HL-60 towards granulocytes resulted in a significant increase in the expression of the g160 kDa antigen. Previous studies on differentiation in *T. annulata* have drawn general parallels between the processes of differentiation in the parasite and higher eukaryotic cells. The reactivity shown by monoclonal antibody 4H5 against HL-60 during differentiation to granulocytes was used as a sensitive marker to extend these parallel studies. The results indicated that during differentiation of HL-60 there is a reversible phase with respect to the 4H5 epitope and that the ability to differentiate is modulated by conditions which alter the growth rate relative to division. As similar findings have been found in *T. annulata* it is proposed that the basic mechanisms controlling differentiation in higher eukaryotes and protozoan cells may be related, reflecting the operation of a primitive mechanism which has been retained by lower and higher eukaryotes.



## **Declaration**

The experimental work described in this thesis was carried out in the Department of Veterinary Parasitology, University of Glasgow, between October 1993 and February 1997. Except where stated otherwise, the results presented are my own.

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## Abbreviations

ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
bp	base pair
cDNA	complementary deoxyribonucleic acid
dH <sub>2</sub> O	distilled water
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
DNA	deoxyribonucleic acid
kb	kilo base
kDa	kilodalton
RNA	ribonucleic acid
<i>spp.</i>	species
te	tick equivalent
Tris	Tris (hydroxymethyl) aminomethane

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# General Introduction

## 1.1. Introduction

*Theileria* spp. are group of tick borne protozoan parasites responsible for diseases of a wide variety of wild and domestic ruminants. *Theileria* spp. are classified within the sub-phylum Apicomplexa, which also includes other important parasitic genera such as *Babesia*, *Eimeria*, *Plasmodium* and *Toxoplasma* (Levine, 1988). There are six species of *Theileria* which are infective for cattle and domestic buffalo. These species are distinguished by geographical distribution, the morphology of macroschizont and piroplasm stages, pathogenicity, vector species and by indirect immunofluorescence antibody tests (Kimber, *et al.*, 1973). The two most important species both clinically and economically are *T. parva* and *T. annulata*. *T. parva* is transmitted by *Rhipicephalus* species of ticks and causes East Coast fever, while *T. annulata* is transmitted by *Hyalomma* spp. and causes tropical theileriosis (Dolan, 1989). Also significant is *T. sergenti*, which is transmitted by *Haemaphysalis* spp. and is an important pathogen in East Asia. The remaining three species, *T. taurotragi*, *T. mutans* and *T. velifera* are only rarely pathogenic. Other members of the genus include *T. camelensis*, which infects camels, *T. hirci*, which infects sheep and goats, and *T. ovis*, *T. recondita* and *T. separarata*, which are non-pathogenic parasites of small ruminants (Dolan, 1989). This project was concerned with the study of *T. annulata* and subsequent sections will concentrate on this species, however, reference to other species will be made where appropriate.

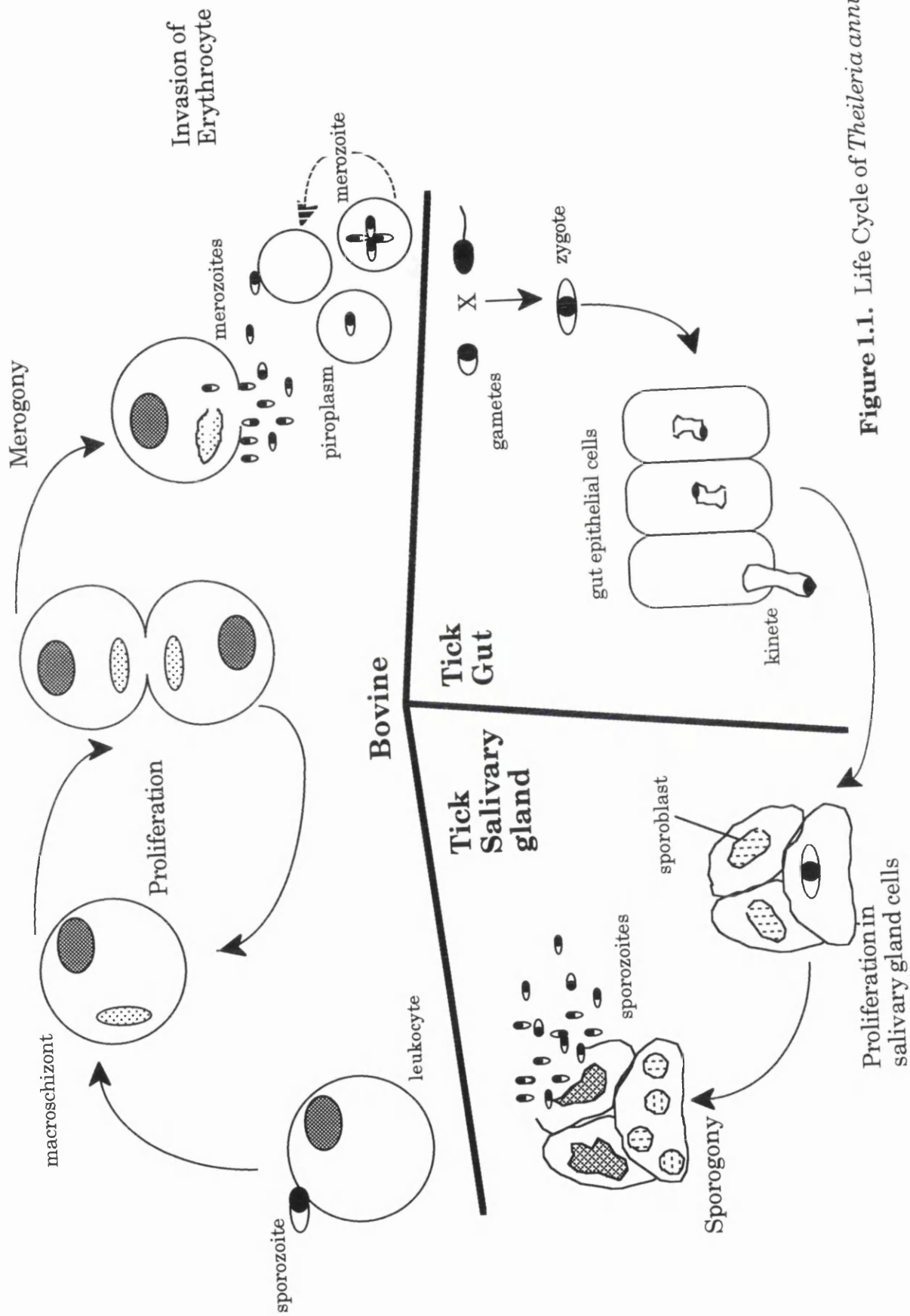
*T. annulata* was first described by Dschunkowsky and Luhs (1904) and is the etiological agent of tropical theileriosis, a debilitating and often fatal

disease. Tropical theileriosis occurs over a vast area stretching from North West Africa and Southern Europe through India and the Middle East to parts of Northern China. Geographical limitations of the disease reflect the availability of a suitable vector species and environmental factors such as temperature (Robinson, 1982). In Africa the disease is restricted to the northern countries and is found in Tunisia, Morocco, Egypt, Sudan, Algeria and Libya. In Europe *T. annulata* is found in the southern countries of Greece, Spain, Italy, Bulgaria and Portugal; with the range of the parasite extending to the Middle East, Southern Russia, India, Pakistan, and China on the Asian continent (Purnell, 1978). Over 200 million cattle are thought to be at risk from the disease, which imposes severe constraints on the production and improvement of livestock in endemic areas (Purnell, 1978). In addition to the cost of treatment, surviving animals often suffer from reductions in milk yield and loss of body weight, as well as abortion and infertility. Such economic losses are especially acute in many developing countries where there is increasing pressure to feed a growing human population and, where domestic animals are used not only for food, but also for tractive power and as a source of fertiliser. Precise losses resulting from this disease are difficult to calculate, however the development of effective, safe and low cost control measures for tropical theileriosis are of great importance to the economy of countries where the disease is endemic.

## **1.2. The life Cycle of *Theileria annulata***

*Theileria spp.* undergo a complex life-cycle alternating between the bovine host and the tick vector (Figure 1). Differentiation from one stage to another is fundamental to the biology of these parasites and facilitates expansion and transmission within and between the host and vector. Asexual multiplication of the parasite takes place at three stages, by





**Figure 1.1.** Life Cycle of *Theileria annulata*.

sporogony in the tick vector and by schizogony and merogony in the bovine host, while sexual reproduction occurs in the tick.

### **1.2.1. Bovine stages**

The bovine host becomes infected with *T. annulata* through the inoculation of sporozoites in the saliva of feeding ticks. Sporozoites invade leukocytes within 5-60 minutes of inoculation, and as many as 15 sporozoites can be internalised within an individual leukocyte after 60 minutes (Jura, *et al.*, 1983). In *T. parva* entry of sporozoites into bovine leukocytes involves a defined series of events, and whilst these have not been as well characterised for *T. annulata*, many similarities exist. Initially the sporozoite recognises and binds to one or more sites on the leukocyte plasma membrane. The process of sporozoite invasion then takes place rapidly, within 15 minutes at 37°C (Shaw, *et al.*, 1991). A close continual junction forms between the sporozoite and the leukocyte membranes. The sporozoite moves inside the host leukocyte by progressive and circumferential "zippering" of the closely apposed membranes (Fawcett, *et al.*, 1982a). While initial recognition and binding of the sporozoites to the lymphocyte membrane is not temperature dependant and can take place at 0-2°C, subsequent zippering and internalisation is, indicating that this is an energy dependant process (Jura, 1984; Shaw, *et al.*, 1991). The separation of host and sporozoite membranes coincide with the discharge of both the microspheres and the rhoptries, with the contents of the microspheres forming a layer of dense fuzzy material 10-15 nm thick on the surface of the sporozoites (Fawcett, *et al.*, 1982a; Shaw, *et al.*, 1991; Williams and Dobbelaere, 1993). The parasite escapes into the host cell cytoplasm as the host cell membrane disintegrates and an orderly array of microtubules surround the sporozoite and the subsequent developing parasite, closely associating with the fuzzy material on the parasite surface (Fawcett, *et al.*,

1984; Fawcett, *et al.*, 1982a; Shaw, *et al.*, 1991; Stagg, *et al.*, 1980). Unlike related protozoans such as *Toxoplasma* and *Plasmodium*, *Theileria* is not retained inside a parasitophorous vacuole (Mehlhorn and Schein, 1984).

Conflicting data exists as to whether the sporozoites enter the host cell in any particular orientation. Jura, *et al.* (1983) found that *T. annulata* sporozoites consistently attached to the leukocyte plasma membrane by their basal end, possibly at specific receptor sites, whereas Mehlhorn and Schein (1984) reported that sporozoites entered in any orientation. Experiments with *T. parva* have implicated a role for MHC class I in the binding of sporozoites to host leukocytes. Shaw, *et al.* (1991) demonstrated that monoclonal antibodies reactive with MHC class I molecules or with  $\beta_2$ -microglobulin could inhibit sporozoite entry. Additional evidence for the involvement of MHC class I came from experiments using a series of bovine deletion mutant cell lines from which one or both MHC I haplotypes had been lost. Shaw, *et al.* (1995) were able to demonstrate a correlation between the level of MHC class I expression and sporozoite binding. Using a double deletion cell line with less than 5% of the normal class I expression, invasion of sporozoites was reduced by over 95% compared to parental lines. Almost all nucleated cell types express MHC I, however, the highest levels of class I are expressed on the surface of lymphocytes where they constitute approximately 1% of the total plasma membrane proteins (Kuby, 1992). These results implicate a role for binding to MHC I in the entry of sporozoites into lymphocytes where the molecule is present in large amounts, although they cannot fully explain the invasion of other leukocyte types and binding to more specific receptors in addition to MHC I could also be involved. *T. annulata* is known to infect a different leukocyte population to that of *T. parva*, and it is not known whether the binding of *T. annulata* sporozoites to host leukocytes occurs by a similar mechanism. *In vitro T.*

*annulata* has been shown to preferentially infect cells positive for MHC class II, that is cells of the monocyte/macrophage and B cell lineages, though the infection of B cells is much less efficient (Glass, *et al.*, 1989; Spooner, *et al.*, 1989; Spooner, *et al.*, 1988). *T. parva* preferentially infects T cells and in certain circumstances B cells (Baldwin, *et al.*, 1988; Naessens, *et al.*, 1985). However, *in vivo* it has been demonstrated that infected cells stop expressing a number of cell surface markers, including those of B cells and monocytes, making it difficult to determine the precise cell type infected (Glass and Spooner, 1990; Spooner, *et al.*, 1989; Spooner, *et al.*, 1988). The molecular nature of species specificity is unknown, however, the distribution of specific cell receptors could play an important role in the determination of cell tropism and it is also likely that other factors, such as differences in the intracellular environment following invasion could be involved.

In *T. annulata* inhibition of sporozoite invasion has been demonstrated using antibodies directed against an expressed recombinant sporozoite surface antigen (SPAG-1) gene fragment (Williamson, *et al.*, 1989). The predicted polypeptide sequence of SPAG-1 was shown to have homology to a repetitive domain in bovine elastin. As elastin is present on a large range of cell types including macrophages/monocytes it was thought the SPAG-1 could represent the ligand by which *T. annulata* recognises its host cell (Hall, *et al.*, 1992). This was later discounted by Campbell, *et al.* (1994) who showed that *T. annulata* sporozoites infected both elastin receptor positive and negative cell populations. More recently Shaw (1996) was able to inhibit sporozoite entry using a range of treatments known to affect cell adhesion processes, however, as a total inhibition of sporozoite entry could not be achieved, this could indicate that sporozoite binding/internalisation

could involve low affinity interactions between a number of both host and parasite molecules.

Once inside the host leukocyte the sporozoites develop into trophozoites, a transient feeding stage (Jura, *et al.*, 1983), before undergoing nuclear division to form multinucleated macroschizonts. The macroschizonts lie free within the host cell cytoplasm surrounded by a plasma membrane that has no obvious outer coat (Mehlhorn and Schein, 1984; Shaw and Tilney, 1992). Nuclei are scattered randomly throughout the schizont cytoplasm and are surrounded by a typical nuclear envelope (Shaw and Tilney, 1992). Mitochondria are usually found in pairs throughout the cytoplasm, together with conspicuous numbers of membrane free ribosomes and small clusters of polysomes. The schizont cytoplasm contains very few other organelles, with no Golgi apparatus or endoplasmic reticulum (Shaw and Tilney, 1992). Development of the macroschizont induces host cell blastogenesis which leads to the rapid clonal expansion of parasitised cells. During this process, at least *in vitro*, host and parasite cell division occur synchronously (Hulliger, *et al.*, 1964). Recently a *cdc2*-related kinase, with over 60% homology to the p34(*cdc2*)/p32(CDK2) eukaryotic cyclin-dependant kinase has been isolated from both *T. annulata* and *T. parva*. Expression patterns of RNA and protein indicate that the *Theileria* *cdc2*-related kinase probably functions in all dividing stages of the parasite and is likely to play an important role in the regulation of nuclear division (Kinnaird, *et al.*, 1996). During division the parasite becomes associated with the mitotic apparatus in prophase and metaphase, and over this time the DNA is replicated (Irvin, *et al.*, 1982). Daughter parasites are separated by cytokinesis of the host cell during anaphase, but the division of the parasite may be unequal. Cells which may have lost the parasite as a result of unequal division cease to divide after a few days.

The majority of macroschizont infected cells continue to grow and divide. However, a proportion undergo the process of merogony, whereby numerous uninucleate merozoites are formed from the syncytial schizont. Initially, merogony is characterised by structural and organisational changes. An endoplasmic reticulum is formed and the outer surface of the schizont becomes covered by a thick coat (20-25 nm), which persists and is found on the surface of mature merozoites. Changes are also observed in the schizont nuclei where condensation of chromatin and close apposition of the nuclear envelope around part of the nucleus takes places. Nuclei migrate by an unknown mechanism to the periphery of the schizont, such that the modified portion of the nuclear envelope becomes closely associated with the schizont plasma membrane. Mitochondria also become associated with the non-specialised part of the nuclear envelope. Rhoptries appear within the schizont cytoplasm, frequently in small clusters in close proximity to both the nuclear envelope and to fibrous material which ultimately connects both the rhoptries and the nuclear envelope to an inwardly projecting peg from the schizont membrane. Thus, prior to the formation of free merozoites the nucleus, mitochondria, rhoptries and plasma membrane all become interconnected. The next stage is the formation of individual unicellular merozoites by 'budding' from the schizont. As outlined by Shaw and Tilney (1992), this process involves the formation of a microtubule basket which encloses the rhoptry and nuclear complex in plasma membrane. At the end of merogony the host cell contains large numbers of mature merozoites and a residual body. Break down of the host plasma membrane eventually leads to merozoite liberation. As *de novo* synthesis of organelles does not take place, the mature merozoite must contain all the organelles required for the next stage of the life cycle. This is ensured by the interconnection of these organelles to the plasma membrane. Free merozoites (1-2  $\mu\text{m}$  in diameter)

are covered in a 20-25 nm thick coat with a single nucleus and between 3 to 6 rhoptries (Mehlhorn and Schein, 1984). In addition, the cytoplasm contains free ribosomes, 1-2 mitochondria and microspheres. Unlike other members of the Apicomplexa, no clearly defined apical complex or conoid is present.

Very little is known about the entry of merozoites into erythrocytes. Work on *T. sergenti* has suggested that the anterior of the merozoite is involved in the initial attachment to the erythrocyte (Kawamoto, *et al.*, 1990). In direct conflict to this, work by Shaw and Tilney (1995) suggested that merozoite invasion takes place by a mechanism very similar to that described for sporozoites (Shaw, *et al.*, 1991), with recognition and attachment to the erythrocyte occurring in any alignment, without re-orientation of the apical end of the parasite. Following internalisation, the merozoite escapes from the enclosing erythrocytic plasma membrane and this process coincides with the discharge of the rhoptry contents. Host membrane fragmentation occurs and the parasite lies free within the cytoplasm. Unlike the macroschizont, host cell microtubules do not associate with the parasite surface. The entry of *Theileria* merozoites into erythrocytes appears to be different from that of other Apicomplexan zoites. For example, following attachment, *Plasmodium spp.* and *Babesia spp.* re-orientate themselves to position the apical end next to the erythrocyte membrane and entry into the host cell is facilitated by the discharge of the rhoptries (Bannister and Dluzewski, 1990; Igarashi, *et al.*, 1988; Mitchell and Bannister, 1988).

Merozoites appear inside erythrocytes approximately 8 days after infection, with up to 90% of erythrocytes becoming infected. Two general forms; slender, comma-shaped- and ovoid forms are then observed within the erythrocyte. The occurrence of the two forms varies according to species,

with approximately 80% ovoid forms observed in *T. annulata*. Ovoid piroplasm forms are thought to represent gamonts which are pre-adapted for the generation of gamete like stages formed in the gut of the tick, whereas comma shaped forms are thought to undergo division by binary fission (Mehlhorn and Schein, 1984). In this instance, nuclear division is always associated with cellular division so true schizonts do not occur. Occasionally four merozoites form a tetrad or "Maltese-cross" arrangement and it has been suggested that this represents a significant dividing form of *T. annulata in vivo* (Mehlhorn and Schein, 1984). The merozoites generated within the erythrocyte by this process are identical to those produced by intraleukocytic schizogony, leading to the suggestion that these forms could be responsible for the re-invasion of erythrocytes (Conrad, *et al.*, 1985). However, the exact role of this stage in maintaining persistent infection remains unclear, clonal expansion of the parasite population has already taken place in the preceding life cycle stages and it would seem that further expansion of the parasite population is unnecessary. The bovine stage of the life cycle is completed when the piroplasm stage is ingested by the tick vector during feeding.

The processes of sporogony and merogony all involve cellularisation from a syncytium and it has been proposed that these processes take place by almost identical steps. Mature sporozoites and merozoites are morphologically similar and contain the same organelles packaged in an identical manner. Shaw and Tilney (1992) concluded from this that the pattern of cellularisation evolved once, and proposed that the most logical way of repeating the same developmental process at different points of the parasite life cycle would be to use the same cassette of structural genes combined with the novel expression of genes determining stage specific differences such as surface coat composition.



### 1.2.2. Tick Stages

*T. annulata* is transmitted transtadially by ticks of the genus *Hyalomma*. During their life cycle the ticks may feed on two or three different hosts, separated by periods in vegetation or within crevices inside livestock housing (Robinson, 1982). Approximately 4 days after feeding and ingestion of piroplasms, ray-bodies are formed. This appears to be initiated by the lysis of the erythrocyte within the tick gut (Cowdry and Ham, 1932; Mehlhorn and Schein, 1984). Ray bodies are spindle shaped with a thorn like apex and several flagellar protrusions 8-12  $\mu\text{m}$  in length. From these macro-and micro- gametes develop undergoing syngamy to form zygotes in the gut of the tick. The zygotes enter the epithelial cells lining the gut where they differentiate into club-shaped motile kinetes. The kinetes then move through the haemolymph to the salivary glands where they penetrate the salivary alveoli and transform into fission bodies (Schein, *et al.*, 1975; Schein, *et al.*, 1978). The parasites undergo nuclear division to form a multinucleate syncytium, from which large numbers of sporozoites develop (Fawcett, *et al.*, 1982b; Fawcett, *et al.*, 1985; Mehlhorn and Schein, 1984). Mature sporozoites are secreted in the saliva of feeding ticks, completing this stage of the life cycle (Purnell and Joyner, 1968).

### 1.3. Pathogenesis

The clinical symptoms of tropical theileriosis are associated with both the intracellular schizont and the intraerythrocytic piroplasm stage. Following tick infestation there is an incubation period of between 9 and 24 days. Expansion of the infected lymphoblast population causes swelling of the lymph nodes draining the site of initial sporozoite inoculation. This is soon followed by hypertrophy of lymph nodes throughout the body, and schizonts

are also found in the liver and spleen. There is a fever of 41°C, which stays high until recovery or death. In the latter stages there is loss of appetite with corresponding weight loss and a general lack of condition. Diarrhoea, often containing blood and mucous, is seen together with a sporadic cough and discharge from the eyes and nose (Barnett, 1977). There is progressive leucocytosis with leukocyte counts rising from a normal of approximately 10 000/mm<sup>3</sup> to between 12 000 and 36 000/mm<sup>3</sup> (Barnett, 1977), followed by leucopenia (Preston, *et al.*, 1992; Srivastava and Sharma, 1980). In *T. parva* infections only slight lowering of the red cell count is seen in the latter stages of the disease, whereas *T. annulata* infections are characteristically accompanied by severe haemolytic anaemia caused by the removal of infected erythrocytes by the liver and spleen (Hooshmand-Rad, 1976). Death occurs in susceptible animals approximately 20 days post infection.

Recently, it has been proposed that the diverse clinical symptoms observed during the course of an infection could be a result of cytokines produced in response to the parasite (Preston, *et al.*, 1993). Administration of macrophage derived tumour necrosis factor alpha (TNF- $\alpha$ ) has been shown to induce cachexia, pyrexia and leucopenia, symptoms similar to those observed in tropical theileriosis (Beutler and Cerami, 1986; Ulich, *et al.*, 1987). Preston, *et al.* (1993) found that *T. annulata* macroschizont infected cells produce interferon- $\alpha$  (INF- $\alpha$ ), which in turn could lead to the synthesis of IFN- $\gamma$  by lymphocytes and TNF- $\alpha$  by macrophages *in vitro*. Such interactions were postulated as being able to set off a cascade of cytokine production leading to the notable clinical features of tropical theileriosis, and provided evidence of a link between the immune response of the host and the pathogenicity of the parasite.

#### 1.4. Epidemiology and Diagnosis

Tropical theileriosis has a pronounced seasonality in sub-tropical countries where the tick vectors are more active in the summer, however, this is less noticeable in countries where the climate permits the ticks to be active all year round (Uilenberg, 1981). The simplest tests for diagnosis of theileriosis are, observation of clinical symptoms and the detection of macroschizonts and piroplasms in Geimsa stained blood smears. The availability of monoclonal antibodies to the sporozoite (Williamson, *et al.*, 1989), macroschizont (Shiels, *et al.*, 1986a; Shiels, *et al.*, 1986b) and piroplasm (Glascodine, *et al.*, 1990) stages of the parasite could allow the development of enzyme linked immunosorbent assays (ELISA) to detect the production or release of parasite antigens during active infections. Kachani, *et al.* (1992) used sera from immunised cattle to determine that a piroplasm antigen was the most suitable for the development of a sensitive ELISA, because it exhibited low non-specific detection by normal sera and high post-infection values. The potential also exists for the use of DNA probes to detect *Theileria* parasites within clinical samples. d'Oliveira, *et al.* (1995) have recently developed a polymerase chain reaction (PCR) assay using species specific primers against the gene encoding the Tams1 major merozoite surface antigen. This assay successfully detected the carrier state in cattle infected with *T. annulata* from four geographically distinct stocks. DNA probes have also been used in epidemiological studies. Ben-Miled, *et al.* (1994) used two piroplasm genomic DNA probes to show polymorphism within *T. annulata* macroschizont infected cell lines and piroplasms isolated from 17 sites in Tunisia.

## 1.5. Bovine Immune Response

In regions where the parasite is found the mortality due to theileriosis is low (about 5%) in the indigenous *Bos indicus* cattle. Most are infected as calves and have a minimal reaction followed by recovery (Brown, 1990). However, the more productive European taurine cattle, often introduced into endemic areas, are highly susceptible to *T. annulata* infection, with mortality rates of 40-70% (Neitz, 1957). Cattle which recover from *T. annulata* infections are immune to challenge from the same stock of parasite and are often immune to challenge from other stocks from different geographical regions (Dyer and Tait, 1987; Gill, *et al.*, 1980; Gill, *et al.*, 1981a; Preston, 1981). The bovine immune response has been reviewed by a number of authors Brown (1990), Hall (1988) and Tait and Hall (1990) and can be divided into humoral and cell mediated responses.

### 1.5.1. Humoral response

Antibodies to both the macroschizont and piroplasm infected stages of the parasite have been observed, but several observations suggest that they do not play a role in protective immunity. Immune bovine sera does not recognise the surface of schizont infected leukocytes in *T. annulata* (Shiels, *et al.*, 1989) or *T. parva* (Creemers, 1982; Duffus, *et al.*, 1978) and this is also the case for piroplasm infected erythrocytes (Hall, 1988). In addition, immune serum used in passive transfer experiments has been unsuccessful in preventing disease (Muhammed, *et al.*, 1975). Calves born to hyperimmune dams have been found to be fully susceptible to challenge from the same *T. parva* stabilate, despite significant levels of anti-*Theileria* antibodies being present in the colostrum (Cunningham, *et al.*, 1989). Experiments where lysates (Wilde, 1967) or inactivated schizonts (Pipano, *et al.*, 1977) have been used in immunisation animals have shown no

protective response. Similarly immunisation attempts using piroplasm extracts have also been unsuccessful (Cowan, 1981; Wagner, *et al.*, 1974). Therefore, it has been generally concluded from these observations that a protective immune response is not directed against the two major pathogenic stages.

In contrast, *in vitro* studies have shown that the invasion of leukocytes by sporozoites can be prevented by immune serum (Gray, *et al.*, 1981). Similar findings were reported by Musoke, *et al.* (1982) and Preston and Brown (1985), both of which showed that the neutralising activity could be increased by repeated sporozoite challenge. As invasion occurs rapidly, sporozoites are only exposed to the host immune system for a relatively short amount of time. Repeated sporozoite challenge is therefore probably required in order to elicit high enough antibody titres to prevent invasion. Preston and Brown (1985) demonstrated that, as well as inhibiting sporozoite invasion, anti-sporozoite antibodies were able to reduce the rate that trophozoite infected cells transformed into proliferative macroschizont infected cells. Inhibition of invasion has been demonstrated using antibodies directed against the expressed product of a gene encoding the sporozoite surface antigen (SPAG-1) (Williamson, *et al.*, 1989). The ability of immune sera to block sporozoite invasion means that sporozoite antigens have potential for use in recombinant subunit vaccine development.

In addition to sporozoites, merozoites are a second stage in the life cycle of the parasite which is exposed to the host immune system. Antibody responses to merozoites have been detected in cattle recovering from *T. annulata* infection (Irvin and Morrison, 1987; Pipano, 1974) and opsonisation by immune sera of free merozoites has been reported by Ahmed, *et al.* (1988). However, there is no conclusive evidence that *in vivo*

anti-merozoite antibodies contribute to host immunity (Hall, 1988; Irvin, 1985). A major merozoite surface antigen of 30 kDa has been characterised (Dickson and Shiels 1993; Glascodine, *et al.*, 1990), but the lack of a suitable *in vitro* assay has meant that the ability of anti-merozoite antibodies to prevent invasion of erythrocytes has remained largely untested. Work on *T. sergenti* however, has shown that the passive transfer of a monoclonal antibody directed against a molecule related to the 30 kDa antigen in *T. annulata* resulted in protection of calves against challenge by sporozoites (Tanaka, *et al.*, 1990).

### **1.5.2. Cell Mediated Response**

As a result of the general lack of evidence for involvement of the humoral response in the control of *Theileria spp.* infections, work has concentrated on the role of the cell mediated immunity. Evidence suggests that the macroschizont infected leukocyte is the main target of the immune response in *T. annulata*. Animals can be immunised with schizont infected cells from either an infected animal (Brown, 1990; Hall, 1988) or by cell lines attenuated in tissue culture. Cytotoxic peripheral blood lymphocytes (PBLs) which lyse *T. annulata* infected lymphoblasts *in vitro* have been found in calves recovering from challenge with sporozoites (Samed, *et al.*, 1983; Singh, *et al.*, 1977). Preston, *et al.* (1983) showed that recovery of calves from *T. annulata* infection was associated with the appearance of cytotoxic cells in the blood and lymph nodes and the disappearance of schizont infected cells. When immune cattle were challenged, two sequential cytotoxic cell populations were produced. Lysis of schizont infected cells by the first population was found to be restricted by bovine MHC (BoLA) and was postulated therefore, to be analogous to cytotoxic T cells. The second cytotoxic cell population contained both MHC-restricted T cells and non-MHC restricted natural killer (NK) like cells. This study also

demonstrated that, during a primary infection cytotoxic cells could be detected only in the circulation and lymph nodes of calves destined to recover. The parasite specific cytotoxic cell responses of immunised animal are transient and therefore other effectors of the immune response may be involved. A third type of cellular response was identified when adherent cells, presumed to be macrophages, were isolated from the peripheral blood of immunised calves and shown to exhibit strong cytostatic effects on both BoLA matched and mis-matched schizont infected cell lines (Preston and Brown, 1988). The mechanism by which infected lymphocyte proliferation was inhibited appeared to be mediated by soluble factors, as inhibition still took place when contact between macrophages and target cells was prevented by a 0.45  $\mu$ M filter.

## **1.6. Control Measures**

There are three major approaches to the control of tropical theileriosis; vector limitation, chemotherapy of infected cattle and host vaccination. In recent years a number of authors have reviewed the approaches undertaken to the control of theileriosis; Brown (1990), Dolan (1989) and, Tait and Hall (1990).

### **1.6.1. Vector Control**

To limit tick infestation cattle can be dipped or sprayed with acaricides, such as amitraz or butocarb, once a week (Urquhart, *et al.*, 1987). As *Theileria* occurs in areas where other tick borne diseases are also present, this method has the advantage of potentially controlling more than one disease. However, there are several major disadvantages associated with the use of acaricides. Apart from their high cost and the potential damage done to the environment, highly organised programs of acaricide application

are required to ensure that all cattle are treated. Long term use can lead to resistance in ticks, and various domestic and wild animals can act as reservoir hosts to maintain an infected tick population. A significant reduction in the tick population can also be brought about by improvements to animal housing which remove sites harbouring ticks (Pipano, 1989).

### **1.6.2. Chemotherapy**

The analogues of the naphthoquinones, menoctone (McHardy, *et al.*, 1976), parvaquone (Gill, *et al.*, 1981b) and buparvaquone (McHardy, *et al.*, 1985) are most effective in the treatment of theileriosis. These drugs interfere in a specific manner with the electron transport chain of the parasite. The drugs destroy macroschizonts within leukocytes and buparvaquone is also effective against piroplasmic stages (McHardy, *et al.*, 1983; McHardy, *et al.*, 1985). Both parvaquone and buparvaquone have no significant side effects, but the latter would seem to be more effective in the treatment of *T. annulata*, with an approximately 88% recovery rate (Hashemi-Fesharki, 1991). Halofuginone has also been used but it is toxic to the animals at levels close to the therapeutic dose (Schein and Voigt, 1979). In the past, tetracycline was used in large doses (Mallick, *et al.*, 1987) and the 8-aminoquinoline derivatives, pamaquin and primaquine were also used, but were effective only against the piroplasm stages (Zhang, 1987). As with acaricides, the major limitation on the use of therapeutic drugs is the high cost of diagnosis and treatment.

### **1.6.3. Vaccines**

The most widely used control measure against *T. annulata* infection is the attenuated macroschizont vaccine. Attenuation involves the repeated passage of schizont infected lymphocytes in culture until pathogenicity is lost. It is not clear whether attenuation is a result of increased



immunogenicity or decreased pathogenicity, although the latter seems more likely. The exact mechanism by which attenuation is brought about is unknown, but a number of changes accompany the loss of virulence. These include alterations in production of host cell proteases (Baylis, *et al.*, 1992), and a reduction in the ability to differentiate into merozoites (Pipano, 1989). As the number of passages increases, virulence can be tested by inoculation into susceptible cattle. The number of passages required to fully attenuate the parasite varies between 30 and several hundred, depending on the isolate (Pipano, 1989). Vaccinated cattle show good protection against natural infections and against heterologous challenge (Gill, *et al.*, 1980; Hashemi-Fesharki, 1988). In addition, unlike the related *T. parva*, there is no histocompatibility barrier to immunisation with *T. annulata* infected cell lines, making it unnecessary to BoLA match donor cell lines with recipient animals (Innes, *et al.*, 1989). Once attenuation has been achieved the infected leukocytes are cryopreserved in aliquots at  $-70^{\circ}\text{C}$ , which can either be used to vaccinate cattle directly or to initiate further cultures. The usual vaccine dose is approximately  $5 \times 10^5$  cells. The immunity conferred by some attenuated cell lines often does not prevent the formation of piroplasms in the blood, leading to the development of a carrier state by the host. These piroplasms are thought to arise either from new infections via feeding ticks or by differentiation of a limited number of vaccine line derived schizonts (Pipano, 1992). The vaccine lines can be cultured in fermentors of 10-20 litres making it simple and relatively inexpensive to produce. The major draw backs to the use of this vaccine are the short shelf life, approximately 1 week at  $20^{\circ}\text{C}$  or 1 month at  $4^{\circ}\text{C}$ , and the constraints imposed by the need for frozen transport of cryopreserved vaccine (Brown, 1990). A second approach to vaccination is the method of infection and treatment (reviewed in Brown, 1990). A defined dose of sporozoites, harvested from ticks and maintained as a cryopreserved stabilate, is

inoculated into cattle and the infection blocked by the administration of theilericidal drugs such as tetracycline, buparvaquone or parvaquone (Bansal and Sharma, 1989; Dhar, *et al.*, 1987; Dhar, *et al.*, 1988). Originally developed for protection against East Coast fever, where the use of attenuated cell line vaccination is not practical, this treatment is expensive and therefore seldom used to control *T. annulata* infections.

Identification of specific parasite antigens which are potential targets for a protective immune response could lead to the development of recombinant vaccines. Isolating the genes encoding these antigens and expressing them *in vitro* using recombinant DNA techniques could lead to the development of a subunit vaccine. A number of antigens on various life cycle stages have been identified as potential candidates for inclusion in a molecular vaccine, these include the surface antigens of the sporozoite, the macroschizont infected cell and the merozoite.

Most research in this area has centred on the sporozoite surface antigen SPAG-1. This molecule was initially isolated by the screening of cDNA libraries with monoclonal antibodies which were able to inhibit sporozoite invasion in an *in vitro* assay (Williamson, *et al.*, 1989). The entire SPAG-1 gene was later isolated and sequenced (Hall, *et al.*, 1992). SPAG-1 was recently shown to be similar to the *T. parva* sporozoite antigen p67 (Knight, *et al.*, 1996; Nene, *et al.*, 1992), as cross reacting epitopes were identified within the C- and N- termini of the molecules. In recent vaccine trials, immunisation with recombinant p67 induced sporozoite neutralising antibodies and provided protection to 6 out of 9 animals against homologous sporozoite challenge (Musoke, *et al.*, 1992). Similarly, a C terminal region of SPAG-1 was shown to be the location of major neutralising determinants (Boulter, *et al.*, 1994). When expressed as a fusion protein, one particular

recombinant antigen produced high titres of neutralising antibodies in immunised cattle and showed some evidence of protection to sporozoite challenge (Boulter, *et al.*, 1995).

The generation of an effective protective immune response against merozoite stages of the parasite would have the potential of eliminating the carrier state of immune cattle and therefore, limit transmission to the tick vector. However, in regions of endemic stability it is unclear that blocking transmission would be an advantage if tick transmission contributes to immunity. A major polypeptide which is strongly recognised by bovine immune sera has been identified on the surface of merozoites (Glascodine, *et al.*, 1990). This molecule was shown to have two forms of variant molecular mass (30- and 32- kDa) and the genes *Tams1-1* and *Tams1-2* corresponding to these allelic variants were recently cloned (Dickson and Shiels, 1993). In order to assess the potential use of Tams1 as a component for inclusion in a subunit vaccine, both these genes have been expressed in *Escherichia coli* and *Salmonella typhimurium* (d'Oliveira, *et al.*, 1996) and preliminary trials have indicated that these recombinant antigens may provide protection against challenge (d'Oliveira, *et al.*, 1997). Immunoblotting studies have demonstrated that the Tams1 antigen shows extensive antigenic diversity. Evidence was obtained that some of the diversity was conferred by secondary modification of the polypeptide, and it is interesting to note that there is significant amino acid diversity at putative N-linked glycosylation sites both within and between species (Shiels, *et al.*, 1995). However, there is no direct evidence that the molecule is glycosylated and the amino acid diversity may play a more direct role in the generation of antigenic variants. As six possible Tams1 alleles have been detected in a single geographical population of *T. annulata*, the

variation displayed by this antigen must be an important consideration when considering its inclusion in a possible subunit vaccine.

### **1.7. Parasite Control of Host Cell Gene Expression**

One of the most fascinating aspects of the biology of *Theileria* is the unique ability of the macroschizont stage of *T. annulata* and *T. parva* to immortalise the bovine leukocyte in which it resides. *In vivo*, this causes a rapid expansion in the number of infected cells prior to differentiation into the merozoite stage; while *in vitro*, immortalisation has permitted the establishment of macroschizont infected cell lines which proliferate indefinitely. Infected cell lines can be isolated from infected lymphoid tissue (Malmquist and Brown, 1970), from peripheral blood mononuclear cells of infected animals or by the *in vitro* infection of leukocytes with sporozoites isolated from infected ticks (Brown, *et al.*, 1981; Brown, *et al.*, 1973; Walker and McKellar, 1983). The mechanism by which *Theileria sp.* initiates and controls host cell blastogenesis is as yet poorly understood but, as one of the most interesting aspects of parasite biology, this area is the focus of much research. Infected leukocytes display many of the characteristics common to other transformed cells, such as altered cell surface phenotype (Baldwin, *et al.*, 1988; Shiels, *et al.*, 1989; Shiels, *et al.*, 1986b), short generation time (16 to 25 hours) and tumour formation (Fell, *et al.*, 1990; Irvin, *et al.*, 1975). However, unlike other transformed cell lines, the immortalisation process is reversed following removal of the parasite (Brown, *et al.*, 1989). A number of changes associated with immortalisation by the parasite have been found, and it has been suggested that some of these may be involved in the transformation process. For *T. parva*, documented alterations include increases in the levels of casein kinase II (CKII), the transcription factor NF- $\kappa$ B, interleukin 2 (IL-2) and

the IL-2 receptor (IL-2R), while in *T. annulata* changes in protein kinase patterns (Dyer, *et al.*, 1992), altered expression of matrix metalloproteinase and the transcription factor AP-1 (Baylis, *et al.*, 1992; Baylis, *et al.*, 1995), increases in IL-2R expression (Herrmann, *et al.*, 1989) and antigenic differences on the surface of the infected host cell (Shiels, *et al.*, 1986b) have been reported.

### **1.7.1. IL-2 and IL-2R**

The role of IL-2 and IL-2R was reviewed recently by Williams and Dobbelaere, (1993). Proliferation of lymphocytes *in vitro* is normally dependant on the presence of lymphokines. However, when bovine lymphocytes are infected with *T. parva* or *T. annulata* they become independent of exogenous growth factors (Brown and Logan, 1986). Dobbelaere, *et al.* (1988) were able to show that *T. parva* infected lymphocytes continuously secrete a growth factor that is essential for their proliferation *in vitro*. Dilution of this secreted factor in cultures results in growth retardation which can be restored by the addition of human recombinant interleukin 2 (IL-2). Elimination of the parasite from the cytoplasm of infected cells using theileriocidal drugs resulted in the termination of growth factor secretion, followed by growth arrest and reversion of the infected cells to the morphology of resting lymphocytes. Using PCR it was demonstrated that a wide range of *T. parva* infected cell lines expressed IL-2 mRNA, albeit at low level when compared to concanavalin A stimulated PBLs (Heussler, *et al.*, 1992). In related studies, biologically functional high affinity IL-2 receptor (IL-2R) was found to be constitutively expressed on the surface of T and B cells infected with *T. parva*, and was associated with the continued presence of the parasite (Coquerelle, *et al.*, 1989; Dobbelaere, *et al.*, 1988; Dobbelaere, *et al.*, 1990). These experiments lead to the suggestion that proliferation of *T. parva*

infected lymphocytes might occur through an IL-2/IL-2R autocrine loop. Inhibition experiments using anti-IL-2 antibodies however, suggested that IL-2/IL-2R mediated autocrine growth might not occur in all cases. Several *T. parva* infected cell lines were found to be significantly inhibited by the addition of anti-IL-2 antibodies to cultures, but at least one B cell line was not inhibited at all and a CD4<sup>+</sup> CD8<sup>+</sup> T cell line was only marginally inhibited (Dobbelaere, *et al.*, 1991). This suggests that other growth factors and receptors may be involved in proliferation, although an alternative explanation is that IL-2 molecules could associate with IL-2R before reaching the cell surface (Dobbelaere, *et al.*, 1991), as has been shown for the growth factor IL-3 (Browder, *et al.*, 1989).

The high affinity IL-2R receptor is composed of a 55 kDa  $\alpha$  chain (Tac antigen) and a 75 kDa  $\beta$  chain (Minami, *et al.*, 1992). Both subunits are able to bind IL-2 with different affinities; the  $\alpha$  subunit has low affinity; the  $\beta$  subunit has intermediate affinity; and the  $\alpha\beta$  heterodimer has very high affinity. Resting T cells express only the  $\beta$  subunit and do not proliferate in response to IL-2. Activation of T cells induces expression of the Tac antigen, which then associates with existing  $\beta$  subunits to form the high affinity IL-2R. IL-2 secreted by activated T cells then binds to the IL-2R, setting up an autocrine signal transduction pathway resulting in cell proliferation. Binding of IL-2 by IL-2R normally results in the internalisation of the IL-2/IL-2R complex, leading to the down regulation of IL-2R, unless expression of the Tac antigen is renewed by continued antigen stimulation (Roitt, *et al.*, 1993). In *T. parva* infected cells the IL-2/Tac gene is constitutively transcribed, resulting in the continued replacement of receptors and the absence of a requirement for exogenous stimulation for continued proliferation (Coquerelle, *et al.*, 1989). Recently, antisense RNA expression was used to assess the role of the IL-2R  $\alpha$  chain on proliferation.

*T. parva* infected T cells were permanently transfected with one of two vector constructs carrying either the sense or antisense sequence for the bovine IL-2R  $\alpha$  chain, under the control of an inducible promoter. A strong reduction in IL-2R  $\alpha$  chain mRNA was observed only in antisense transfected cells and was accompanied partial inhibition of infected T cell proliferation. Some IL-2R  $\alpha$  chain was still detected in antisense transfected cells and might account for the lack of complete inhibition reported (Eichhorn and Dobbelaere, 1995). These results are consistent with the observations made from the experiments with anti-IL-2 antibodies and support the postulation that proliferation is only mediated in part by the IL-2/IL-2R autocrine loop. Other cytokines may be involved in *Theileria* induced proliferation and it has recently been shown that IL-1, IL-6 and IL-10 are also produced by *T. annulata* infected cells (Brown, *et al.*, 1995).

Ivanov, *et al.* (1989) showed that lymphocytes infected with *T. parva* contained constitutively high levels of the transcription factor NF- $\kappa$ B in both the nuclear (activated form) and cytoplasmic (inactive form) fractions. They demonstrated that the slow killing of the parasite, which left host cells intact, resulted in the rapid loss of activated NF- $\kappa$ B from nuclear fractions and a slower loss from cytoplasmic fractions, where the factor was inactivated by binding to its inhibitor I- $\kappa$ B. They concluded that, increased synthesis of the inactive forms and the maintenance of high levels of the activated form were induced by the presence of the parasite. NF- $\kappa$ B was originally identified as the protein controlling the expression of the  $\kappa$  light chain immunoglobulin (Ig) gene. DNA sequences similar to the motifs recognised by NF- $\kappa$ B have been identified in many genes including IL-2 and IL-2R. Thus, as suggested by Heussler, *et al.* (1992), the high levels of NF-

$\kappa$ B could be responsible for the constitutive expression of IL-2 and IL-2R in parasitised lymphocytes.

The role of IL-2 and IL-2R in *T. annulata* infected cells is less clear. *T. annulata* infected cells were shown not to require or produce IL-2 (Ahmed, *et al.*, 1987). In later work, IL-2R  $\alpha$  chain expression and IL-2 responsiveness was demonstrated for some, but not all *T. annulata* infected cell lines (Ahmed, *et al.*, 1992; Dobbelaere, *et al.*, 1990). Binding of radiolabelled IL-2 was abolished after elimination of the parasite by the application of buparvaquone, but unlike *T. parva* infected cells, IL-2R  $\alpha$  chain mRNA was not reduced, and in some cases increased following elimination of the parasite. *T. annulata* infected cells have been shown to predominantly express the intermediate affinity IL-2R (Herrmann, *et al.*, 1989) and indirect evidence suggested that buparvaquone interfered with the expression of the IL-2R $\beta$  chain on the surface of *T. annulata* infected cells (Ahmed, *et al.*, 1992). Differences in IL-2 response and IL-2R expression may be explained by the fact that *T. annulata* and *T. parva* infect cells of different lineages, and consequently the precise mechanisms employed by each species to induce proliferation of the host cell may vary. Alternatively, the alterations to cytokine pathways may be a secondary event of the immortalisation process and reflect the expression pattern of the uninfected host cell type.

### **1.7.2. Altered patterns of phosphorylation**

There is a well established association between cellular transformation and altered protein kinase activity, particularly on tyrosine residues (Hunter and Cooper, 1985; Hunter and Sefton, 1980). Many transforming oncogenes have also been found which encode protein kinases (De Feo, *et al.*, 1981). In agreement with other transformed cell lines, increases in protein kinase



activity have been reported in both *T. annulata* (Dyer, *et al.*, 1992) and *T. parva* (ole-MoiYoi, *et al.*, 1993) infected cells. Work in this field has concentrated on the role of casein kinase II (CKII) in *T. parva* transformed cells, which was recently reviewed by ole-MoiYoi, *et al.* (1993) and ole-MoiYoi, *et al.* (1992).

CKII is a ubiquitous serine-threonine specific protein kinase composed of two catalytic subunits ( $\alpha$  or  $\alpha'$ ) and two regulatory  $\beta$  subunits. The nucleotide and amino acid sequences are highly conserved throughout evolution and it has been suggested that the CKII enzyme may play a critical role in cell function (Seldin and Leder, 1995). CKII may be regulated during the cell cycle (Carroll and Marshak, 1989), and has been postulated to be involved in the regulation of multiple cellular metabolism and gene expression pathways (Pinna, 1990). Rapidly proliferating cells, some human leukemias (Friedrich and Ingram, 1989) and solid tumours (Munstermann, *et al.*, 1990) show elevated levels of CKII. Increases in the transcriptional, translational and functional levels of bovine CKII have been observed in *T. parva* infected cells, implicating this enzyme as an important element in signal transduction pathways activated during immortalisation (ole-MoiYoi, *et al.*, 1993). The *T. parva* genome has been found to encode the  $\alpha$  sub unit, but no evidence that the parasite encodes the  $\beta$  subunit has been found to date (ole-MoiYoi, *et al.*, 1992). This is also the case for *Drosophila* (Saxena, *et al.*, 1987) and *Zea mays* (Dobrowolska, *et al.*, 1992) and has lead to the hypothesis that the parasite CKII  $\alpha$  subunit could insert into the parasite plasma membrane or be secreted into the host cell cytoplasm at some phase of the cell cycle (ole-MoiYoi, 1995). From these locations the parasite enzyme could then phosphorylate bovine substrates without being subjected to normal regulatory controls.

Evidence for the role of CKII in signal transduction pathways leading to transformation has been provided in recent experiments using transgenic mice (Seldin and Leder, 1995). When the catalytic sub unit of CKII was expressed in the lymphocytes of transgenic mice, no detectable increase in the total amount of CKII $\alpha$  protein or in the phosphotransferase activity of the enzyme was observed. However, the transgenic mice exhibited a high predisposition to the development of lymphomas, implying that neoplastic transformation induced by the dysregulated expression of a critical regulatory element in signal transduction or cell cascade pathways was not necessarily associated with elevated concentrations of that element. When the CKII $\alpha$  transgene was subsequently co-expressed with its *in vivo* substrate, *c-myc* (Luscher, *et al.*, 1991), the animals rapidly developed murine perinatal leukemia. In addition, co-expression of the two oncogenes disrupted the regulation of lymphocyte gene expression, as analysis of cells from one bi-transgenic tumour showed the presence of surface molecules representing two normally exclusive lymphoid differentiation pathways.

The work described above demonstrates that CKII has a role in the pathogenesis of lymphoproliferative disorders. It is intriguing to find that CKII is also over expressed in *Theileria* infected cells which cause a disease of similar pathology. However, as rapidly dividing cells commonly show elevated levels of CKII activity, it remains to be seen whether this enzyme plays a direct role in the process of parasite induced transformation.

### **1.7.3. Metalloproteinases**

Infection of bovine leukocytes with *T. annulata* has been shown to result in the expression of nine novel proteinases (Baylis, *et al.*, 1992). Inhibition studies using divalent metal ion chelators characterised these proteinases as belonging to the metalloproteinase class, and one of these proteinases

was subsequently identified as the bovine homologue of human metalloproteinase 9 (MMP9) (Baylis, *et al.*, 1995). More recently, a number of highly specific tissue inhibitors of metalloproteinases were used in substrate gel assays to remove the activities of these enzymes, providing conclusive evidence of their identity (Adamson and Hall, 1996). Matrix metalloproteinases are a family of homologous enzymes capable of degrading components of the extracellular matrix. *In vivo* they are involved in tissue remodelling during wound healing and in embryogenesis (Agren, *et al.*, 1992; Reponen, *et al.*, 1992). They have also been implicated in a number of pathogenic processes, such as the passage of tumour cells across basement membranes during metastasis (Alvarez, *et al.*, 1990), and in the tissue damage found during rheumatoid arthritis (Okada, *et al.*, 1987). In the bovine macroschizont infected cells can form discrete tumour-like foci throughout the body (Irvin and Morrison, 1987), and disseminating solid tumours have been observed in SCID mice injected with *Theileria* infected bovine cells (Fell, *et al.*, 1990). MMP9 in particular has been implicated in the metastasis of malignant tumours, which has lead to the conjecture that the metalloproteinases up-regulated by the parasite have a role in the metastatic behaviour of infected cells. Using a Matrigel™ *in vitro* invasion assay, macroschizont infected cells have been shown to be capable of crossing reconstituted basement membranes. Furthermore, this metastatic behaviour is almost eliminated when specific inhibitors of metalloproteinases are included (Adamson and Hall, 1996). There is also a marked reduction in the expression of proteases in the attenuated vaccine line ODE, with a corresponding four fold reduction in metastatic potential (Adamson and Hall, 1996; Baylis, *et al.*, 1992). This evidence has lead to the suggestion that metalloproteinases are virulence factors mediating some of the pathological features of tropical theileriosis (Adamson and Hall, 1996).

In related work, elevated expression of the transcription factor AP-1 has been found to be associated with the presence of the parasite in infected cells (Baylis, *et al.*, 1995). The human MMP9 gene is known to contain AP-1 binding sites in the region up-stream of the transcription start site (Huhtala, *et al.*, 1991), and it may be that this is a key factor in the up-regulation of MMP9 following infection. Increases in the levels of AP-1 activity are also involved in the immediate early response to mitogenic stimuli, with evidence for a role in both G<sub>0</sub>/S and G<sub>1</sub>/S cell cycle progression (Muller, *et al.*, 1993). Baylis, *et al.* (1995) suggested that infection of resting cells by the parasite could result in the progression from G<sub>0</sub> to S phase and that parasite dependant proliferation could be caused by the continued stimulus of G<sub>1</sub> to S phase progression.

AP-1 is composed of heterodimers of the Fos and Jun family of proteins or of Jun homodimers, which bind only weakly to DNA (Curran and Franza, 1988). The composition of the AP-1 observed in *T. annulata* infected cells is unknown, although *c-fos* mRNA levels are increased (Baylis, *et al.*, 1992). The mechanism by which the parasite brings about up regulation of AP-1 remains to be elucidated, but a number of hypothesis have been proposed. These include the possibility that the AP-1 complex contains parasite derived factors, or that the parasite increases AP-1 levels by the secretion, or surface expression, of kinase or phosphorylase like factors which stimulate signal transduction pathways. A more general mechanism could be mediated by induced changes to host cellular components such as cAMP, Ca<sup>+</sup> or redox potential, which in turn alter AP-1 levels (Baylis, *et al.*, 1992).

#### 1.7.4. Antigenic differences

Immortalisation by *Theileria* is known to bring about phenotypic alterations to the surface of the host cell (Shiels, *et al.*, 1986b). One such alteration is the expression of a polypeptide with a variable molecular mass ranging from 99 kDa to 125 kDa, depending on cell type (Shiels, *et al.*, 1989). This molecule is assumed to be of host origin, as it is not recognised by immune bovine serum (Shiels, *et al.*, 1989), but is recognised by a monoclonal antibody, 4H5. This polypeptide is the focus of work presented in this thesis and is discussed in greater detail in Chapter 2.

The alterations which have been described above are probably secondary events and not directly involved in the immortalisation process. However, theoretical connections can be made between some of these events which could give clues to the possible mechanisms involved in immortalisation. Both of the transcription factors discussed in relation to the control of MMP9 (AP-1) and IL-2/IL-2R (NF- $\kappa$ B) gene expression have roles in the regulation of cytokines known to be produced by *Theileria* infected cells (Baeurele, 1991; Chen and Rothenberg, 1993). In addition, components of AP-1, c-Fos and c-Jun are able to functionally synergise with the 65 kDa subunit of NF- $\kappa$ B, leading to a potentiation of biological function and enhanced transcriptional activity at both  $\kappa$ B or AP-1 enhancer-dependant promoters (Stein, *et al.*, 1993, and references therein). Levels of AP-1 activity could also possibly be affected by CKII. It has been shown that CKII phosphorylation results in the inhibition of c-Jun activity (Lin, *et al.*, 1992), and that CKII can also induce *c-fos* expression via the serum response element pathway (Gauthier-Rouviere, *et al.*, 1991).

### 1.8. Stage Differentiation in *T. annulata*

In general, the life cycles of protozoan parasites consist of a progressive series of differentiation steps from one life cycle stage to the next. These differentiation steps are essential for the establishment and transmission of parasite populations. Terminal differentiation does not take place, instead parasites cycle through a set number of distinct differentiation events, one of which involves the formation of sexual stages. The understanding of the molecular mechanisms which control differentiation in protozoan parasites is an important aim of research into these organisms. Such an understanding could provide new ways of controlling life cycle events and lead to better control and treatment of the diseases caused by these parasites. In addition, the elucidation of the molecular mechanisms which control differentiation in parasitic protozoa might provide valuable insights into similar mechanisms operating in higher eukaryotic systems.

Although many factors are known to induce differentiation in the parasitic protozoa, very little is known about the underlying mechanisms involved. In some instances it has been suggested that an exogenous factor(s) could be the source of a signal to differentiate. In *Trypanosoma brucei* differentiation from long slender to short stumpy bloodstream forms in the mammalian host is thought to ensure vector transmissibility. Factors which trigger this differentiation event are unknown, but as differentiation to the short stumpy form occurs following the attainment of a certain parasite population density, it has been suggested that trypanosomes produce a specific factor which regulates differentiation. A model of short stumpy differentiation has been proposed based on the constant production of such a factor. *In vivo*, as the parasite population increased, factor concentration would also increase until a threshold value, sufficient to

trigger differentiation was eventually reached (Giffin and McCann, 1989; Hesse, *et al.*, 1995; Pays, *et al.*, 1993; Seed and Sechelski, 1989). However, despite extensive research in this field no such differentiation factor has been identified in any parasitic protozoan system to date.

Parasites have complex life cycles where the transition between hosts and/or different life cycle stages is fundamental. Alterations in the parasitic environment as a result of these transitions has been suggested as the source of stress factors which could induce differentiation. For example, in *Leishmania spp.* transformation of promastigotes (insect form) to amastigotes (mammalian form) takes place during phagocytosis by host macrophages (Chang and Dwyer, 1976). Since approximately two pH units difference exists between the promastigote and the amastigote environment, physiological changes in pH have been suggested to induce differentiation. In support of this Zilbertstein, *et al.* (1991) were able to show that expression of an *L. major* amastigote specific antigen could be induced in promastigotes following exposure to acidic pH *in vitro*.

The most striking example of environmental change would seem to take place in parasites which transfer between a poikilothermic insect vector and a homeothermic mammalian host. The obvious temperature difference between vector and host has lead to suggestions that a heat shock response could be responsible for the initiation of differentiation. Stress factors, such as increased temperature, rapidly activate heat shock genes resulting in the extensive synthesis of heat shock proteins (Hsp) and a rapid decrease in transcription of most other genes (Ashburner and Bonner, 1979). In *T. brucei* mRNA transcripts of Hsp 70 and Hsp 83 were found to be 25 to 100 times more abundant in bloodstream forms compared to the procyclic insect stage (Van der Ploeg, *et al.*, 1985). However, unlike other eukaryotes the

expression of heat shock genes did not interfere with cell proliferation, indicating that the heat shock response may have a different role in these parasitic protozoa. Alterations in growth conditions have also been shown to mimic the heat shock response as bloodstream forms of *T. brucei* cultured at 37°C with fibroblast feeder layers retained heat shock gene expression and did not de-differentiate when shifted to 22°C (Van der Ploeg, *et al.*, 1985). In *Leishmania* temperature elevation appears to be a significant factor in the induction of differentiation *in vitro* (Eperon and McMahon-Pratt, 1989; Hunter, *et al.*, 1982; Shapira, *et al.*, 1988). However, heat shock proteins do not seem to play a role in the regulation of this response as no correlation is found between their expression and differentiation (Shapira, *et al.*, 1988; Zilbertstein, *et al.*, 1991). Thus, although elevated temperature is commonly found to induce parasite differentiation *in vitro*, it is unclear whether heat shock proteins have a primary role the control of this process.

One of the most obvious clinical symptoms associated with tropical theileriosis is a fever of 41°C, which develops early in infection and remains until recovery or death (Barnett, 1977). This observation lead to the supposition that the high temperatures produced during the course of infection could provide the stimulus for differentiation from the macroschizont to the merozoite stage. This hypothesis was supported by Hulliger, *et al.* (1966) who showed that *in vitro*, differentiation could be induced by an elevation in culture temperature from 37°C to 41°C during a six day period. The exact nature of the part played by elevated temperature was unknown, but it was noted that at elevated temperatures host cell division was slower or completely arrested, while parasite division was unaffected, resulting in the production of large parasites containing many nuclei.



The onset of fever in infected animals is known to be dose dependant, therefore if the formation of merozoites and subsequent appearance of piroplasms was dependant on temperature, this would also be dose dependant. In a series of experiments it was shown that the appearance of piroplasms occurred as a function of time and was not directly linked to the infective dose or temperature (Jarrett, *et al.*, 1969). Animals were exposed to different numbers of infected ticks and the kinetics of parasite replication followed by daily estimation of macroschizont, merozoite and piroplasm numbers. The results demonstrated that the onset of fever correlated with the time at which approximately  $7 \times 10^9$  schizonts were produced and that the initial dose determined the timing of fever. As the percentage of schizonts present with a given number of nuclei on any particular day was the same, irrespective of the dose, logarithmic growth of the parasites was found to correlate with schizonts containing 1-6 nuclei, while the appearance of piroplasms was found to correlate with schizonts containing 12 to 24 nuclei. Similar findings concerning the increase in the number of schizont nuclei were also found *in vitro* (Moulton, *et al.*, 1971). Thus, the appearance of piroplasms in infected animals was found to be time-dependant and lead to the theory that differentiation of the schizont was brought about following a fixed number of cell divisions.

This so called mitotic clock hypothesis was also suggested to be involved in higher eukaryotic differentiation (Raff, *et al.*, 1988; Temple and Raff, 1986). Normally oligodendrocyte progenitor cells in the developing rat optic nerve differentiate to post-mitotic oligodendrocytes over several weeks (Skoff, *et al.*, 1976). However, when cultured *in vitro* the cells stop dividing and undergo premature differentiation within two to three days (Skoff, *et al.*, 1976). The normal timing of oligodendrocyte progenitor cell differentiation

could be restored *in vitro* by the addition of platelet derived growth factor (PDGF) (Raff, *et al.*, 1988). All the descendants of a single progenitor cell were found to differentiate after the same number of cell divisions when cultured in the presence of PDGF. From these results it was suggested that differentiation occurred by means of a clock which allowed a maximum number of cell divisions to take place before the onset of differentiation, and that the inclusion of PDGF *in vitro* had the effect of re-setting the normal timing of differentiation. Later studies disproved this theory when it was found that differentiation could be influenced by cytokines which directly modulated the proliferative capabilities of the cell. Thus, premature differentiation *in vitro* occurred as a result of the loss of proliferative capacity which could be restored by the addition of PDGF (Raff, *et al.*, 1988) or basic fibroblast growth factor (bFGF) alone (Bogler, *et al.*, 1990). While co-operation between PDGF and (bFGF) promoted continued proliferation and removed the ability of the progenitor cells to differentiate (Bogler, *et al.*, 1990). As cells cultured in the presence of both PDGF and bFGF clearly underwent more mitotic divisions than cells grown in medium supplemented either PGDF or bFGF alone, a strict correlation between the number of divisions and differentiation was not found.

Established *Theileria* infected cell lines cultured at 37°C *in vitro* undergo unlimited proliferation without differentiation. This would suggest that like higher eukaryotic cells, the mitotic clock hypothesis is too simplistic to explain merozoite differentiation. The observation that merozoite formation in infected cell lines cultured at elevated temperatures was associated with decrease or arrest of host cell division, but left parasite division unaffected, lead to the suggestion that the inhibition of host cell division could be involved in the differentiation process. Fritsch, *et al.* (1988) addressed this hypothesis by examining the effect of various drugs on the formation of

merozoites *in vitro*. Colchicine, which inhibits spindle formation during cell division, was found to completely inhibit the mitosis of the host cell with a concomitant increase in the number of schizont nuclei. However, no increase in merozoite production was seen. From these experiments it is possible to conclude that an increase in the number of parasite nuclei *per se* was not sufficient to induce differentiation. However, these experiments were only conducted at 37°C and it was unclear whether the drugs were inhibiting a differentiation event induced by elevated size. Alternatively, it is possible that additional factors are required in conjunction with an increase in the number of parasite nuclei to induce differentiation.

Further studies on stage differentiation in *Theileria* were aided greatly by the isolation of cloned cell lines with either enhanced or diminished abilities to differentiate *in vitro* at 41°C (Shiels, *et al.*, 1992). It has already been established that the early stages of differentiation towards merozoite formation are characterised by an enlargement of the macroschizont and an increase in the number of nuclei. In cloned cell lines it was found that the number of nuclei and the size of the macroschizont was greatly increased during the first 6 days of culture at 41°C in the differentiation enhanced cell line, whereas no comparable size increase was seen in the differentiation diminished cell line (Shiels, *et al.*, 1992). Thus, it was concluded that the increase in parasite nuclear division and growth, coupled to a decrease in parasite cell division as a result of declining host cell division, leads to the substantial enlargement of the schizont to a predetermined size or condition that triggers differentiation.

Having established that the proliferative potential of the cell was an important factor in the ability of *Theileria* infected cells to differentiate, Shiels, *et al.* (1992) went on to demonstrate that differentiation was not an

immediate response to temperature elevation. A series of pulse experiments were carried out, where cultures of the differentiation enhanced cell line were exposed to 41°C for differing periods of time before being returned to 37°C. In order for significant differentiation towards merozoites to take place, a period of at least 24 hours at 41°C was required, and the longer cultures were maintained at the elevated temperature the greater was the extent of differentiation. However, if cultures were not taken to a particular point before return to 37°C they were able to reverse the differentiation process. Thus, the longer they were at 41°C the more likely they were to reach a threshold point where return was not possible. From these results it was concluded that differentiation towards merozoites was a two step process comprising of an initial reversible phase followed by full commitment to irreversible differentiation. In more recent work Shiels, *et al.* (1994) were able to define the temporal order of some of the molecular changes associated with differentiation by using monoclonal antibodies and gene probes to macroschizont and merozoite specific polypeptides. Levels of Tams1 and of the 117 kDa rhoptry antigen Tar 1 were shown to increase dramatically between days 4 and 6 at 41°C. Conversely, the levels of a major macroschizont antigen were shown to decrease over the same time period. Using pulse experiments similar to those described previously (Shiels, *et al.*, 1992) they were also able to show that low level expression of the Tams1 antigen was reversible in the early stages of differentiation. In addition, small numbers of cells were present in these cultures which continued to react with increasing intensity to the anti-Tams1 antibody following return to 37°C. These cells were thought to represented a sub population of cells which had passed through the commitment point during the pulse procedure and so were unable to revert to an undifferentiated state at 37°C.

Re-cloning of the differentiation enhanced cell line resulted in the isolation of cell lines with reduced abilities to differentiate. As both cell lines were shown to have identical genotypes, this was not a result of the presence of a mixed clonal types in the original cell line. In the differentiation diminished cell line occasional parasites were observed which reacted intensely with both the anti-Tams1 and the anti-Tar1 antibodies, demonstrating that the loss in ability to differentiate was not absolute. These results indicate that the observed differences in abilities to differentiate between the two cell lines were probably not due to major genetic change and could be result of epigenetic differences.

The level of up-regulation of Tams1 during the reversible phase of differentiation was found to be higher in the differentiation enhanced cell line. This lead to the hypothesis that the underlying basis determining commitment to differentiation was a quantitative one and this was used as the basis of a stoichiometric model of stage differentiation. In this model differentiation occurs when the level or activity of factors which control gene expression build up to a concentration threshold which triggers differentiation. Preceding this is a reversible phase whereby the build up of factors is below the threshold levels required to commit the cell to differentiation. Removal of the inducer during this time would result in the failure of the cell to reach the commitment threshold, therefore, re-establishing the undifferentiated state. Inherent asynchrony is an inevitable consequence of such a stochastic model of differentiation, as cells become committed to differentiate in a random fashion linked to how near or far they are from the commitment threshold.

Parallels can be seen between the model of stage differentiation in *Theileria* proposed by Shiels, *et al.* (1994) and differentiation events which occur in

both other protozoan parasites and in higher eukaryotic systems. Similar relationships between differentiation and reduced proliferation have been reported. For example, in *T. gondii* factors which affect the proliferative capacity of the tachyzoite are known to induce the formation of bradyzoites (Bohne, *et al.*, 1994; Soete, *et al.*, 1994). Similarly, in higher eukaryotic cells in addition to the oligodendrocyte model discussed, many other examples have been documented. The induction of differentiation in erythroleukemia cells by DMSO has been shown to be directly associated with a reduction in proliferative capacity (Gusella, *et al.*, 1976) and in myogenesis withdrawal from the cell cycle is tightly coupled to differentiation (Rastinejad and Blau, 1993).

Differentiation in other protozoan parasites is known to involve a step where the parasite becomes irreversibly committed to differentiate. For example, in *T. brucei* differentiation from the bloodstream form to the procyclic form can be induced *in vitro* by a combination of cold shock to 27°C and the addition of citrate/*cis*-aconitate to the incubation medium (Czichos, *et al.*, 1986), and is accompanied by major changes in the polypeptide composition of the *Trypanosome* protein coat. These changes occur within a 4-12 hour period and are reversible within this period if the inducer is removed, indicating that changes to the surface coat precede commitment to full differentiation (Ehlers, *et al.*, 1987; Pays, *et al.*, 1993). In *P. falciparum* Bruce, *et al.* (1990) showed that individual schizonts within erythrocytes produced either only asexual parasites or gametocytes and postulated that commitment to a particular developmental pathway took place prior to invasion. In higher eukaryotes, DMSO induced differentiation of either the human leukemic cell line HL-60 towards granulocytes (Tarella, *et al.*, 1982) or of erythroleukemia cells (Gusella, *et al.*, 1976) has been shown to involve

an initial reversible phase with a minimum exposure time of 12-18 hours required to bring about commitment to differentiation.

Asynchrony of differentiation with respect to time can also be observed in a number of protozoan parasites, such as bradyzoite-tachyzoite interconversion in *T. gondii* (Soete, *et al.*, 1993), in *Leishmania* (Sacks and Perkins, 1984), *T. cruzi* (Heath, *et al.*, 1990), and *T. brucei* (Duszenko, 1990) as well as in higher eukaryotic cells such as DMSO induced granulocytic differentiation of HL-60 (Tarella, *et al.*, 1982).

Thus, it is possible to find many similarities between the events known to take place during differentiation in *Theileria* and the differentiation processes of both other protozoan parasites and of higher eukaryotic systems. These similarities could indicate that the underlying processes are essentially similar and that by examining these parallels it may be possible to provide insights into the fundamental molecular mechanisms controlling differentiation.

## 1.9. Objectives of this work

The most interesting aspect of *Theileria* biology is the unique ability of the parasite to bring about immortalisation of the host leukocyte in which it resides. Very little is known of the mechanisms employed by the parasite to control this process, although immortalisation is known to be associated with changes in host cell gene expression. A starting point to the further understanding of this process would be the investigation of how infection by the parasite leads the up-regulation of host molecules. The infection associated molecule recognised by monoclonal antibody 4H5 (TaHBL20/125) (see 1.7.4.) is a good candidate gene with which to examine the process of parasite controlled host cell gene expression. One of the primary objectives of this thesis was to isolate the gene encoding this polypeptide and examine its function and regulation at the molecular level in *Theileria* infected cells. During the preparation of this thesis, work was published concerning the up-regulation by the parasite of another host encoded gene, MMP9 and the possible role of the transcription factor AP-1 in its regulation (Baylis, *et al.*, 1995) (see 1.7.3.). In view of this, a secondary aim of this work was to compare the regulation pathway of TaHBL20/125 with that of MMP9 to investigate if any general relationship existed between these events in the parasitised host cell. As TaHBL20/125 is probably host derived, the polypeptide might also be expressed in cells of a related lineage to those infected by *T. annulata*. A third aim of this thesis therefore, was to examine cells of the monocyte/macrophage cell lineage for the possible presence of a related molecule. If this was found to be the case investigations could be made into the modulation of expression of this polypeptide in a non-parasitised system which could have relevance to the mechanisms employed by the parasite to induce expression during infection. Modulation in the expression of genes often accompanies cell lineage



differentiation, so a fourth aim of this thesis was to look for modulated expression of the molecule recognised by monoclonal antibody 4H5 during the lineage differentiation of higher eukaryotic cells. If such modulated expression was found, the final aim of this thesis was to compare the initiation and progression of differentiation processes in *Theileria* and with those of higher eukaryotes.

## **1.10. Summary of work presented**

Chapter 2: describes the further characterisation of the molecule recognised by monoclonal antibody 4H5 in *T. annulata* infected cells and the relationship between expression and infection by the parasite.

Chapter 3: describes the characterisation of a 160 kDa molecule recognised by monoclonal antibody 4H5 in the human leukemic cell line HL-60 and the modulation of its expression during differentiation *in vitro*.

Chapter 4: describes investigations of the process of granulocytic differentiation in HL-60 and the parallels that exist between differentiation in *T. annulata* and HL-60.

Chapter 5: describes the strategies used to attempt to clone the molecule recognised by monoclonal antibody 4H5 from *T. annulata* infected cells.

Chapter 6: contains a general discussion of the findings of this thesis.

## **Chapter 2**

## **Characterisation of an infection associated molecule from *T. annulata* infected cells**

### **2.1. Introduction**

In common with many other transformed cell lines, *Theileria* infected lymphoblasts have been shown to undergo infection associated changes, some of which have been implicated in the process of immortalisation (see 1.7.). Alterations in the expression of surface membrane antigens often accompany cell transformation (Anderson, *et al.*, 1978; Burridge, 1976). It is not surprising therefore, that alterations to the phenotype of the *Theileria* infected cell surface have been observed (Shiels, *et al.*, 1986b). Using a series of monoclonal antibodies raised against *T. annulata* (Hissar) infected lymphocytes, Shiels, *et al.* (1986b) were able to identify several infection associated antigens by indirect immunofluorescence assay (IFA). Three monoclonal antibodies (4H5, 1E12 and 2A6) were found to recognise antigens on the surface of the schizont infected cell. These antibodies failed to recognise any antigen on normal peripheral blood lymphocytes (PBLs) or on proliferating blasts derived from Concavalin A (Con A), poke weed mitogen (PWM), phytohemagglutinin (PHA) or lipopolysaccharide (LPS) simulated lymphocytes. This suggested that the antigen detected was not commonly found on a sub population of lymphocytes or generally associated with blast formation. These results also provided the first direct evidence that infection by *Theileria* induced novel antigens on the surface of infected lymphocytes.

The three monoclonal antibodies were used to immunoprecipitate surface radiolabelled molecules from extracts of different infected cell lines. 1E12 and 2A6 failed to immunoprecipitate any polypeptides, whereas a single

specific polypeptide was precipitated by 4H5. This polypeptide was found to have a variable molecular mass between different infected cell lines, ranging from 99 kDa in TaHBL20 (Hissar, India), to 116 kDa in TaR (Razi, Iran) and 125 kDa in TaH46 (Ankara, Turkey) (Shiels, *et al.*, 1989). Monoclonal antibody 4H5 failed to detect any polypeptide in BL20, the uninfected counterpart of TaHBL20, providing further evidence that infection by the parasite induced altered expression of polypeptides at the cell surface. These results also demonstrated that alteration in the expression of the 4H5 epitope was a common feature of *T. annulata* infected cell lines from different geographical areas.

In similar immunoprecipitation experiments a second anti-glycoprotein polyclonal antiserum, raised against a lentil lectin (*Lens culinaris*) specific fraction of a TaHBL20 cell extract, identified two infection associated surface molecules with relative molecular masses of approximately 100 kDa and 80 kDa (Shiels, *et al.*, 1989). Two infection independent molecules were also identified by this antibody and were assumed, due to their size and location, to correspond to the heavy and  $\beta_2$ -microglobulin chains of the bovine class 1 (BoLa) antigen. These findings suggested that novel expression of glycoproteins occurs at the surface of the infected cell and confirmed the results of a separate study which characterised the differences between glycoprotein fractions from infected and uninfected cells (Taracha, 1985). In this second study, an infection associated glycoprotein described by Taracha from TaH46 was found to have a similar molecular mass (120 kDa) to that recognised by 4H5. When TaH46 extracts were immunoprecipitated with 4H5 prior to analysis by lectin affinity chromatography, the 120 kDa glycoprotein was lost. Thus, from this and from the similarity in size between the molecule recognised by the anti-glycoprotein antiserum and that recognised by monoclonal antibody 4H5, it

was concluded that the antigen was a cell surface glycoprotein. Glycoproteins comprise the major proportion of molecules found on the cell surface (Gahmberg, 1976) and it is not surprising that the antigen recognised by monoclonal antibody 4H5 should be classified as such. In addition, this classification could also explain the variations in the molecular mass of this molecule, as a number of other cell surface glycoproteins have been shown to display similar variation due to differential glycosylation (Hemler and Strominger, 1982).

Serum from immune bovines has failed to detect infection associated molecules on the surface of the host cell. This implies that such molecules are either host derived and are therefore not recognised by the bovine or, that they are parasite derived and either non-antigenic (having no B cell epitopes) or masked by host cell molecules. Creemers (1982) suggested that molecules induced on the cell surface by the parasite could be either closely associated to or be subtle modifications of host molecules. A number of host molecules have been identified which show quantitative or qualitative alterations associated with the presence of the parasite (see 1.7.) As the polypeptide recognised by monoclonal antibody 4H5 is not recognised by immune bovine serum, but is clearly antigenic, it would seem likely that it is host derived and is either modified or expressed at higher levels following parasite induced immortalisation of the host cell.

### **2.1.2. Aims**

The aim of the work presented in this chapter was to further characterise the antigen recognised by monoclonal antibody 4H5. Firstly, to establish conditions that would permit visualisation of the antigen, without the need for radiolabelling and immunoprecipitation. This in turn would aid

subsequent manipulations and allow determination of the suitability of the molecule for immunoaffinity purification. Secondly, to conclusively demonstrate that the similarly sized molecule recognised by the anti-glycoprotein polyclonal antiserum and monoclonal antibody 4H5 were the same. Thirdly, to establish if the expression of the molecule recognised by monoclonal antibody 4H5 was directly associated with immortalisation by the parasite or was due to a secondary alteration brought about by long-term cell culture.

## **2.2. Material and Methods**

### **2.2.1. Cell culture**

All cell lines were maintained in culture at 37°C, with 5% CO<sub>2</sub>. Cultures were diluted every 2-3 days to an approximate cell density of 1 x 10<sup>5</sup> cells ml<sup>-1</sup> with RPMI-1640 (Gibco) supplemented with 8 µg ml<sup>-1</sup> streptomycin, 8 units µl<sup>-1</sup> penicillin, 0.6 µg ml<sup>-1</sup> amphotericin B (Fungizone, Gibco), 0.05% sodium hydrogen carbonate and 10% heat inactivated foetal calf serum (FCS), for TaHBL20, or 20% Myoclone FCS, for BL20 and monoclonal antibody hybridomas.

### **2.2.2. Cryopreservation of cell lines**

Cell lines were preserved in liquid nitrogen for long term storage. 10 ml of cell culture was centrifuged at 400 g for 5 minutes and resuspended in 3 ml of medium containing 10% dimethylsulphoxide (DMSO). The resuspended cells were placed into cryotubes, wrapped in cotton wool and frozen in a pre-cooled polystyrene box at -70°C for 24 hours before transfer to liquid nitrogen. To recover cell lines, cryopreservation tubes were thawed rapidly at 37°C and the cells pelleted by centrifugation at 400 g for 5 minutes. Cells were washed twice in pre-warmed medium (37°C) before final resuspension in 5 ml of supplemented medium and transfer to a culture flask.

### **2.2.3. *In vitro* infection of BL20 with *T. annulata* (Hissar) sporozoites**

1 ml of stabilate, stored in liquid nitrogen in 7.5 % glycerol in minimal essential medium (MEM, GibcoBRL) was rapidly thawed in water at 37°C and allowed to equilibrate at room temperature for 10 minutes. Stabilates consisted of whole ground up tick (GUTS) material from ticks infected with *T. annulata* (Hissar) at a concentration of approximately 2 tick equivalents

(te) per ml. After equilibration, 1 ml of RPMI/ 20 % FCS prepared as 2.2.1 was added slowly and allowed to equilibrate for 10 minutes. This procedure was repeated with a further 2 ml of medium to give a volume of 4 ml at 0.5 te ml<sup>-1</sup> and 1.9 % glycerol. 10 ml of rapidly growing BL20 cells were diluted in medium to concentration of 10<sup>6</sup> viable cells per ml and a 24 well Linbro plate was used to set up infections as outlined below;

Infection conditions	Wells 1-4	Wells 5-8
Vol. of stabilate at 0.5 te ml <sup>-1</sup>	800 µl	200 µl
Vol. BL20 at 2 x 10 <sup>5</sup> cells ml <sup>-1</sup>	200 µl	200 µl
Vol. medium	-----	600 µl
Final concentration, te ml <sup>-1</sup>	0.4	0.1

The plate was incubated at 37°C, and after 24 hours 1 ml of pre-warmed medium was added to the first 2 wells of each set. For the second 2 wells from each set, 500 ml of medium was removed from the top of the well and replaced with 1.5 ml of medium containing 10 % FCS. After a further two days, cultures from all four wells of each set were made up to 10 ml with medium containing 20 % FCS, placed into tissue culture flasks and cultured as described 2.2.1.

### 2.2.4. Antisera

Monoclonal 4H5 was raised against the surface of *T. annulata* (TaHBL20) infected lymphocytes (Shiels, *et al.*, 1986b). The polyclonal anti-glycoprotein antiserum was raised in a rabbit against lentil lectin fractionated extracts of *T. annulata* infected lymphocytes (Shiels, *et al.*, 1989). Monoclonal antibody 1C12 was raised against *T. annulata* infected



lymphocytes in the same manner as monoclonal antibody 4H5 (Shiels, *et al.*, 1986b).

## **2.2.5. Immunofluorescence assays (IFA)**

### **2.2.5.1. Indirect**

Preparation of antigen slides for IFA was adapted from the method of Minami, *et al.* (1983). Cell cultures were grown to a density of  $1 \times 10^6$  cells  $\text{ml}^{-1}$ . The cells were pelleted by centrifugation at 400 g for 5 minutes and washed three times in PBS (150 mM NaCl, 16 mM  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ , 4 mM  $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ , 4 mM KCl, pH 7.2.). The washed cells were resuspended at  $5 \times 10^7$  cells  $\text{ml}^{-1}$  and fixed by the dropwise addition of an equal volume of 3.7% formaldehyde in PBS, followed by incubation on ice for 10 minutes. The fixed cells were washed three times with PBS, resuspended at  $1 \times 10^7$  cells  $\text{ml}^{-1}$  and spotted onto Wellcome PTFE Multispot slides (C.A. Hendley, Essex). Slides were air dried and stored at  $-20^\circ\text{C}$  in plastic bags containing silica gel. Slides of fixed cell lines were thawed in acetone at room temperature for 5 minutes, and 20  $\mu\text{l}$  undiluted hybridoma supernatant added directly to each well. The slides were then incubated in a moist box for 30 minutes at room temperature followed by three washes with PBS. After air drying, 20  $\mu\text{l}$  of fluorescein isothiocyanate (FITC) conjugated rabbit anti-species IgG (Sigma) was added to each well. The slides were then incubated as before, washed and then counter stained by immersion in 0.1% Evans blue in PBS for 5 minutes. Slides were mounted in DABCO/glycerol (50% glycerol in water with 2.5% (w/v) 1,4 diazabicyclo (2.2.2.) octane (DABCO), pH 8.0.) and examined for fluorescence by microscopy under the x 50 objective of a Leitz ortholux II transmitted light fluorescence microscope.

#### **2.2.5.2. Cell surface IFA**

Cell cultures at a density of  $1 \times 10^6$  cells  $\text{ml}^{-1}$  were pelleted by centrifugation at 400g for 5 minutes and washed 3 times in an equal volume of ice cold PBS. The cells were resuspended to approximately  $5 \times 10^6$  cells  $\text{ml}^{-1}$ . 20 ml of cell suspension was then incubated with 20 ml of undiluted hybridoma supernatant for 30 minutes at 4°C with occasional mixing. Cells were washed 3 times, as before, in 1 ml of ice cold PBS and resuspended in 20 ml of PBS/10% FCS. 20 ml of FITC conjugated rabbit anti-species secondary antibody was added, and the cells incubated for 30 minutes at 4°C, with occasional mixing. Cells were washed 3 times with 1 ml PBS and resuspended in 20 ml of 0.2% formaldehyde in PBS, and spotted onto multispot slides. Slides were allowed to air dry, before counterstaining by immersion in Evan's blue for 5 minutes. Slides were washed with PBS, mounted in DABCO/glycerol and examined for fluorescence by microscopy as described above.

#### **2.2.5.3. DAPI staining of IFA slides**

After staining with Evan's blue and washing, slides were mounted in DABCO/glycerol containing 4, 6-diamidino-2-phenylindole (DAPI) at 1 mg  $\text{ml}^{-1}$  and p-phenyldiamine at 1 mg  $\text{ml}^{-1}$

#### **2.2.6. Preparation of cell extracts.**

Cells were pelleted by centrifugation at 400 g for 5 minutes, washed three times in PBS, resuspended to a density of  $2 \times 10^8$  cells  $\text{ml}^{-1}$  in pre-chilled NP-40 lysis buffer (150 mM NaCl, 1% Nonidet-P40, 50 mM Tris.HCl, pH 8.0) and incubated on ice for 30 minutes. The insoluble material was removed by centrifugation at 15 800 g for 10 minutes and the supernatant retained. NP-40 extracts were stored at -20°C.

#### **2.2.6.1. Protein quantitation of cell extracts.**

The protein concentration of cell extracts was estimated by a modification of the Bradford assay (Bradford, 1976). In order to remove detergent, proteins were first precipitated from extracts by incubating 20 µl of extract with 150 µl of 11% trichloroacetic acid (TCA) for 30 minutes at room temperature. Insoluble material was pelleted by centrifugation at 15 800 g for 5 minutes and the pellet washed twice with 150 µl 11% TCA. Proteins were solubilized in 10 µl 2 M NaOH and 90 µl PBS and added to 5 ml of freshly diluted Bradfords reagent. 5 x Bradfords reagent was prepared by dissolving 100 mg of Coomassie brilliant blue G-250 (Sigma) in 50 ml of 95% ethanol, followed by the addition of 100 ml of concentrated phosphoric acid and distilled water to final volume of 200 ml. The reagent could be stored at 4°C for up to 6 months. Solubilised protein/Bradfords reagent was incubated for a minimum of 5 minutes to enable colour development to occur, and the absorbance at 595 nm recorded. Concentration of protein in the sample was estimated by comparison with a set of bovine serum albumin (BSA) standards ranging from 20 to 1000 µg ml<sup>-1</sup>. 25 µg or 15 µg total cell extract per lane were typically loaded onto Protean II and mini Protean II acrylamide gels respectively.

#### **2.2.7. SDS-Polyacrylamide Gel Electrophoresis (PAGE)**

SDS-PAGE was carried out according to the method of Laemmli (1970), using the Biorad Protean II and mini Protean II gel electrophoresis systems. The resolving gels varied in acrylamide concentration from 10 to 15%, depending on the molecular mass range of the polypeptides being analysed. For the Protean II apparatus, 30 ml of a 10% resolving gel was used routinely. The gel was composed of; 11.9 ml dH<sub>2</sub>O, 10 ml acrylamide mix (30% acrylamide, 0.8% N-N Bis-methylene acrylamide, Scotlab), 7.5 ml of 1.5 M Tris.HCl, pH 8.8 and 300 µl 10% SDS. Polymerisation was

initiated by the addition of 300  $\mu$ l freshly made 10% ammonium persulphate (APS) and 12  $\mu$ l tetra-methyl-1,2-diaminoethane (TEMED, Sigma) . After pouring the gel was overlaid with water saturated 2-butanol and allowed to set for approximately 60 minutes.

Once the resolving gel had polymerised, the overlay was poured off and the top of the gel rinsed with dH<sub>2</sub>O. 12 ml of a 4% stack gel was then poured on top of the resolving gel. The stack gel composed of; 8.20 ml dH<sub>2</sub>O, 2.03 ml acrylamide mix, 1.5 ml Tris.HCl, pH 6.8 and 120  $\mu$ l of 10% SDS. Polymerisation was initiated by the addition of 120  $\mu$ l 10% APS and 12  $\mu$ l TEMED.

Where Protean II mini gels were used the volumes of resolving and stacking gels were reduced to approximately 10 ml and 3 ml respectively, with the appropriate adjustment in the proportions of individual components.

Samples were electrophoresed using either denaturing conditions; where extracts were prepared in standard sample buffer (500 mM Tris.HCl, pH 6.8, 8% SDS, 40% glycerol, 10% 2-mercaptoethanol, 0.002% bromophenol blue) and heated to 95°C for 5 minutes prior to loading, or non-denaturing conditions; where samples were prepared in non-reducing sample buffer (as standard without 2-mercaptoethanol) and not heated prior to loading. Molecular masses of proteins were estimated by reference to molecular mass markers of high mass range (29-205 kDa, Sigma) or low mass range (14.2-66 kDa, Sigma). Protean II gels were typically electrophoresed overnight in electrophoresis tank buffer (50 mM Tris, 384 mM glycine, 2 mM disodium ethylenediaminetetra-acetic acid (EDTA) and 0.1% SDS) at 50 V; while Protean II mini gels were run for approximately 2 hours at 100 V. Following electrophoresis, a calibration curve of log<sub>10</sub> molecular mass of

standard polypeptides versus distance migrated was plotted and used to determine the molecular mass of the sample polypeptides by extrapolation.

### **2.2.8. Western blotting**

Immunoblotting of polyacrylamide gels (Western blotting) was adapted from (Towbin, *et al.*, 1979). Proteins were first separated on polyacrylamide gels ranging from 10-15% and transferred to nitrocellulose filters (Schleicher and Schuell, BA 85, 0.45  $\mu$ m) in transfer buffer (25 mM Tris, 192 mM glycine, 20 % methanol), using either a Bio-Rad Trans blot cell or mini blot cell at 4°C. Transfer was carried out with a current of 300 mA for 3 hours or 1 hour respectively. Efficiency of transfer was determined by staining the filter for 5 minutes with 0.2% Ponceau-S (Sigma) in 3% TCA, followed by destaining in distilled water. Marker lanes were cut off and the filter was rinsed with wash buffer (10 mM Tris.HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) to remove residual stain. The filter was then incubated for 3 hours at room temperature on a rocking platform in block buffer (as wash buffer supplemented with 5% non fat milk powder, 10% horse serum). After blocking filters were incubated overnight at room temperature in the primary antibody; polyclonal antisera was diluted 1:200 in block buffer and monoclonal antibody supernatant was used undiluted. After incubation, unbound antibody was removed by rinsing filters twice, then washing 2 x 15 minutes in wash buffer. Washed filters were incubated for 2 hours with either alkaline phosphatase conjugated anti-species second antibody (IgG whole molecule, Sigma) at a dilution of 1: 30 000 in block buffer, or peroxidase conjugated anti-species second antibody (Sigma) at 1: 10 000 in block buffer. The filters were then washed as before. Antibody binding was detected in the case of alkaline phosphatase conjugated second antibody by the method of Harlow and Lane (1988), using bromochloroindoyl phosphate/tetrazolium (BCIP/NBT) as the substrate.

This generates a purple precipitate at the site of alkaline phosphatase binding and was terminated, when the reaction was judged to have proceeded sufficiently, by washing the filter in tap water and allowing it to air dry. In the case of peroxidase conjugated second antibody, the ECL method was used (according to the manufacturers instructions, Amersham), to generate chemiluminescence at the site of peroxidase binding. The filters were exposed to autoradiography film (Kodak X-Omat AR) at room temperature for varying lengths of time between 30 seconds and 30 minutes. Film was developed by submerging the film in the dark in Kodak LX24 developing solution for 3 minutes, rinsed in 3 % acetic acid for 30 seconds, fixed in Ilford Hypam fixing solution for 3 minutes and finally rinsed in tap water for 5 minutes.

#### **2.2.9. Periodation of immunoblots**

After Ponceau S staining the filter was washed in 20 mM sodium acetate, pH 5.4, 50 mM sodium chloride, and then incubated in the same buffer with 10 mM sodium metaperiodate (Sigma ) for 60 minutes in the dark. The filter was washed 3 times in dH<sub>2</sub>O, incubated for 20 minutes in 20 mM Tris.HCl, pH 7.2, 150 mM NaCl, 200 mM glycerol and washed 3 times in 20 mM Tris.HCl, pH 7.2, 150 mM NaCl. After this treatment the filter was incubated with block buffer and antibodies as 2.2.8.

#### **2.2.10. Immunoprecipitation**

Immunoprecipitations were performed by incubating 150 µl of NP-40 extract overnight at 4°C with 5 µl of purified 4H5 monoclonal antibody (see 5.2.2.). 50 µl of a 10% (v/v in NP-40 lysis buffer) suspension of Protein-A Sepharose 4 Fast Flow beads (Pharmacia) was added to the samples and the sample tubes incubated on a rotator for 60 minutes at room temperature. The beads were pelleted by centrifugation at 8 000 g for 15

seconds, and non specifically absorbed material removed by washing three times in NP-40 lysis buffer. Bound immune complexes were eluted from the beads by incubation in 25 µl SDS-PAGE sample buffer at 85°C for 10 minutes. Samples were then subjected to SDS-PAGE and Western blotting (see 2.2.7. and 2.2.8.).

## **2.3. Results**

### **2.3.1. Immunofluorescence studies using monoclonal antibody 4H5**

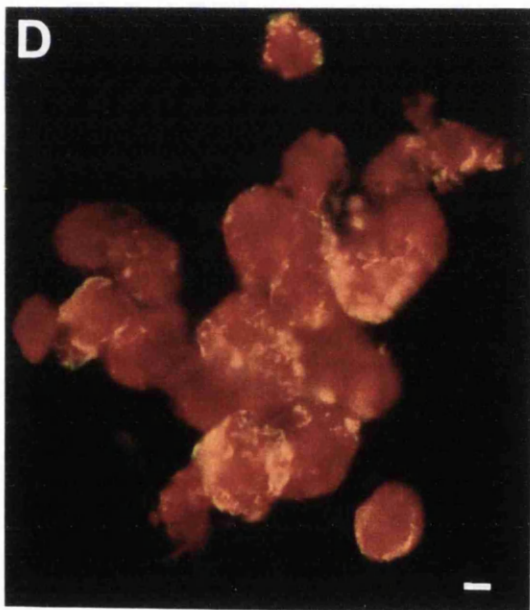
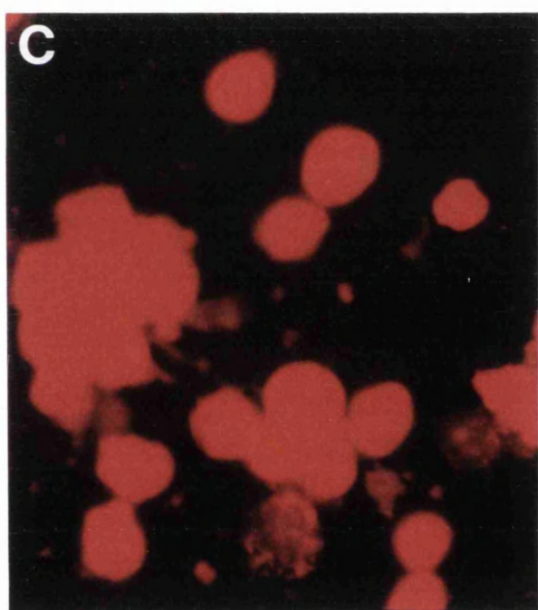
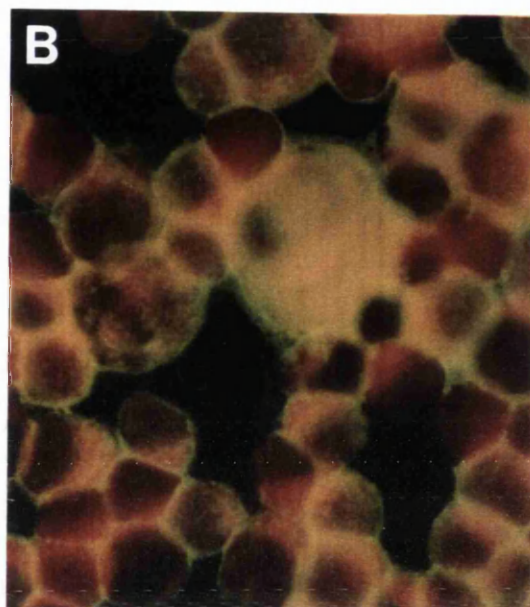
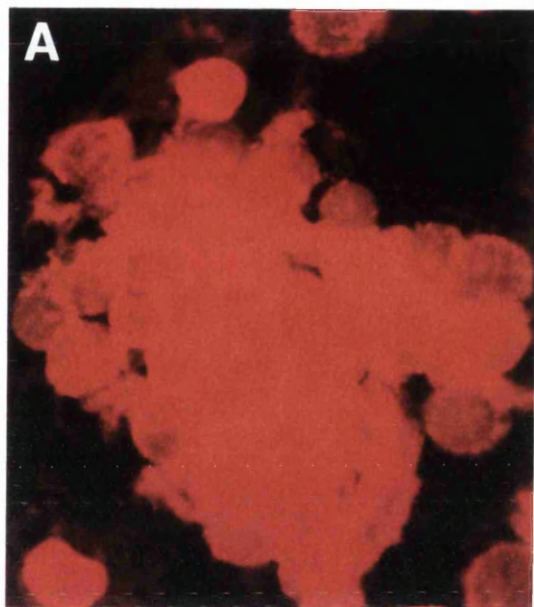
To confirm the antibody reactivity of previous studies, monoclonal antibody 4H5 was used in both indirect and cell surface immunofluorescence assays to investigate the presence of the 4H5 antigen in various cell types (see 2.2.5.). Cells of the bovine lymphosarcoma cell line BL20 were not detected by monoclonal antibody 4H5 whereas, the infected counter part, TaHBL20, showed intense reactivity (Figure 2.1. A and B). In TaHBL20 approximately 75% of the cells showed intense immunofluorescence with this antibody, while the remaining 25% of cells showed either faint or no reactivity. Figure 2.1. shows the pattern of reactivity observed when the same antibody was used in an indirect surface immunofluorescence assay. BL20 cells were not detected by 4H5 (Figure 2.1.C) and a speckled pattern of reactivity was observed on the outside of the infected TaHBL20 cells (Figure 2.1.D). These results indicated that the antigen detected by monoclonal antibody 4H5 in TaHBL20/125 was, at least in part, located on the surface of the infected cell and confirms the infection associated reactivity described previously (Shiels, *et al.*, 1989; Shiels, *et al.*, 1986b).

### **2.3.2. Identification of TaHBL20/125 by immunoblotting**

To characterise the 4H5 antigen at the molecular level, NP-40 extracts (see 2.2.6.) were prepared from BL20 and TaHBL20 cells. Previously this antibody was unable to detect antigen by immunoblotting. To determine whether this was due to recognition of an epitope destroyed by denaturation, electrophoresis was carried out under non-reducing conditions (see 2.2.7.). Under normal PAGE conditions no detectable polypeptides were observed using monoclonal antibody 4H5 to probe immunoblots of either BL20 or TaHBL20 (Figure 2.2. lanes 1 and 2). When



**Figure 2.1.** Immunofluorescence reactivity of monoclonal antibody 4H5 on fixed slide preparations of (A) the uninfected bovine lymphosarcoma line BL-20 and (B) the *T. annulata* infected counter part of A, TaHBL20. Surface immunofluorescence reactivity of monoclonal antibody 4H5 on (C) BL20 and (D) TaHBL20. (Bar = 10  $\mu\text{m}$ )



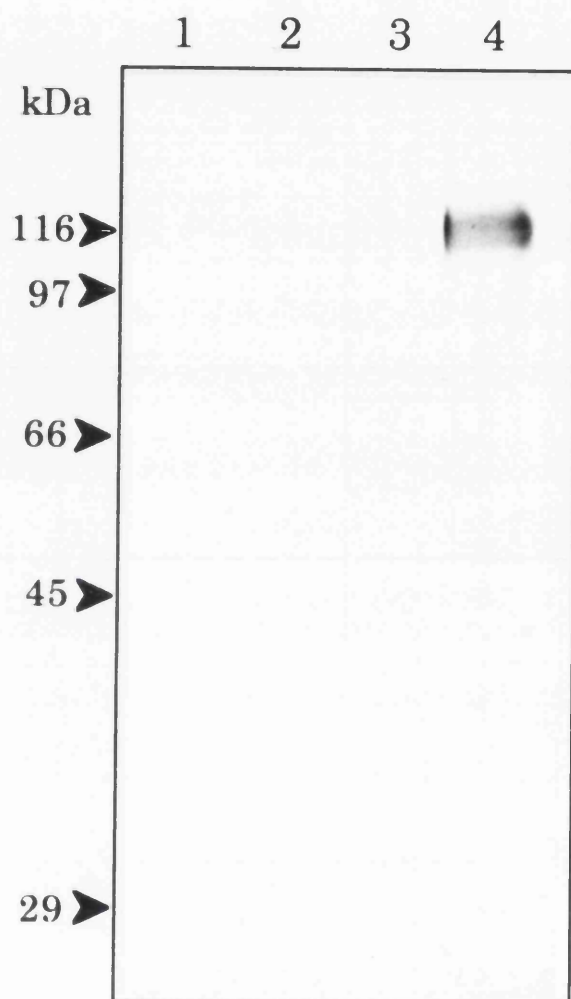
**Figure 2.2.** Analysis of uninfected and infected cell extracts by immunoblotting and probing with monoclonal antibody 4H5 under reducing and non reducing SDS-PAGE conditions.

Lane 1: BL20 reducing

Lane 2: TaHBL20 reducing

Lane 3: BL20 non-reducing

Lane 4: TaHBL20 non-reducing



extracts were run under non-reducing SDS-PAGE conditions, that is 2-mercaptoethanol was omitted from the sample buffer and the samples were not heated to 100°C prior to loading on to the gel, a polypeptide of 125 kDa was clearly detected in TaHBL20 (TaHBL20/125) (Figure 2.2. lane 4). No corresponding band was visible in BL20 (Figure 2.2. lane 3).

Periodate is thought to oxidise vicinal hydroxyl groups on carbohydrate bringing about structural alterations to carbohydrate specific antibody epitopes and removing the reactivity of antibodies which recognise these epitopes. To investigate if monoclonal antibody 4H5 recognises an epitope conferred by secondary modification of the polypeptide, an immunoblot of BL20, TaHBL20 and piroplasm extracts was periodate treated and subsequently probed with monoclonal antibody 4H5 (see 2.2.8. to 2.2.9.). Detection of the 30 kDa Tams1 antigen (see 1.6.3.) by monoclonal antibody 5E1 is known to be periodate sensitive, and served as a positive control in the experiment (Dickson and Shiels, 1993). The Western blot was Ponceau stained and cut into strips. Half of the strips were treated with periodate, the other half were left as untreated controls. The strips were probed with monoclonal antibody 4H5, or in the case of piroplasm extracts, with 5E1. Detection of the 30 kDa antigen by 5E1 was completely abolished by the treatment, indicating that periodate oxidation had been carried out successfully (Figure 2.3. lanes 5 and 6). No specific antigen was detected by monoclonal 4H5 in the uninfected BL20 extracts whether treated with periodate or not (Figure 2.3. lanes 1 and 3). Monoclonal antibody 4H5 detected a single polypeptide of 125 kDa in both the untreated and treated TaHBL20 extracts. However, following treatment with periodate the intensity of TaHBL20/125 increased significantly (Figure 2.3. lanes 2 and 4). This demonstrated that monoclonal antibody 4H5 did not detect an epitope conferred by periodate sensitive carbohydrate moieties.

**Figure 2.3.** Analysis of periodate treated and untreated immunoblots of infected and uninfected cell extracts probed with monoclonal antibody 4H5 or 5E1.

Lane 1: BL20 untreated, probed with 4H5

Lane 2: TaHBL20 untreated, probed with 4H5

Lane 3: BL20 treated with periodate, probed with 4H5

Lane 4: TaHBL20 treated with periodate, probed with 4H5

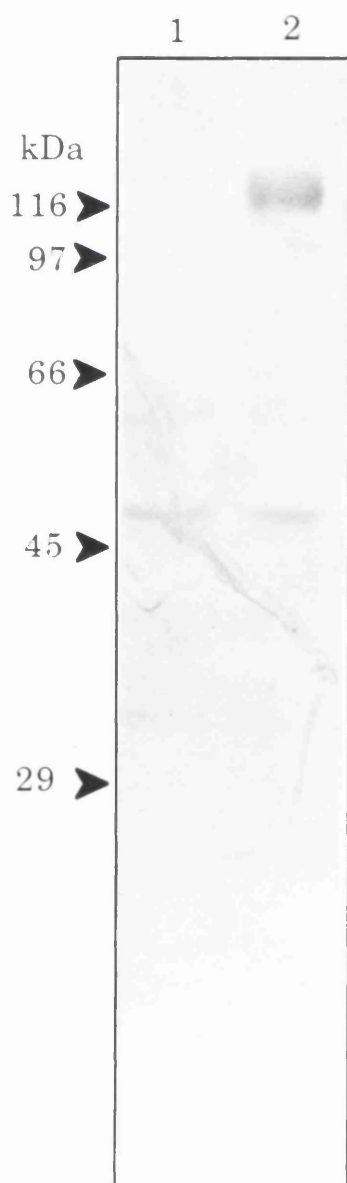
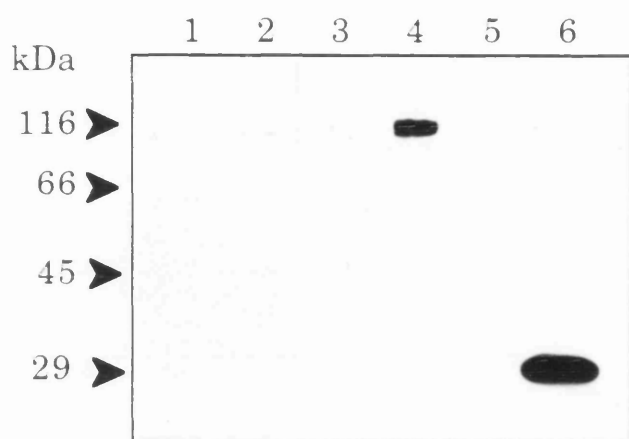
Lane 5: Piroplasm treated with periodate, probed with 5E1

Lane 6: Piroplasm untreated, probed with 5E1

**Figure 2.4.** Immunoprecipitation of infected and uninfected cell extracts with monoclonal antibody 4H5, followed by Western blotting and probing with the anti-glycoprotein polyclonal antiserum.

Lanes 1 BL20

Lane 2: TaHBL20



### **2.3.3. Confirmation of TaHBL20/125 as a glycoprotein**

A second polyclonal antiserum raised against the lentil lectin fraction of infected cells was shown to recognise an infection associated antigen of a similar size to that recognised by monoclonal antibody 4H5 in TaHBL20 (Shiels, *et al.*, 1989). NP-40 extracts were prepared from BL20 and TaHBL20 and immunoprecipitated (see 2.2.10.) using monoclonal antibody 4H5. Following elution of bound immune-complexes, samples were run on a 10% acrylamide gel and immunoblotted using the anti-glycoprotein polyclonal antibody. No specific bands were detected in the uninfected BL20 extract (Figure 2.4. lane 1) and a single band of 125 kDa was detected in TaHBL20 extracts (Figure 2.4. lane 2). These results suggested that both the anti-glycoprotein polyclonal antibody and monoclonal antibody 4H5 recognised the same 125 kDa molecule in TaHBL20 cells, confirming that the 125 kDa antigen was likely to be glycosylated.

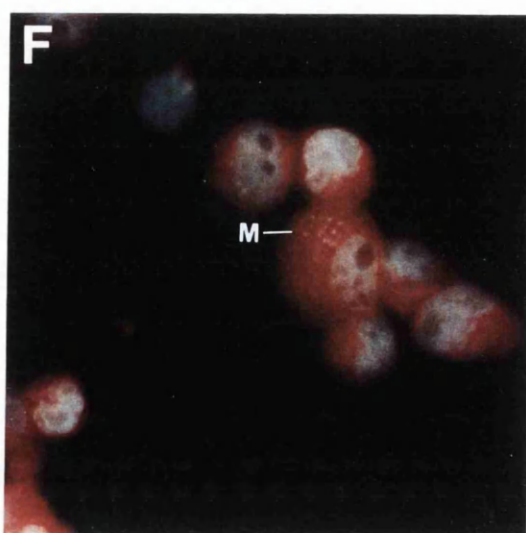
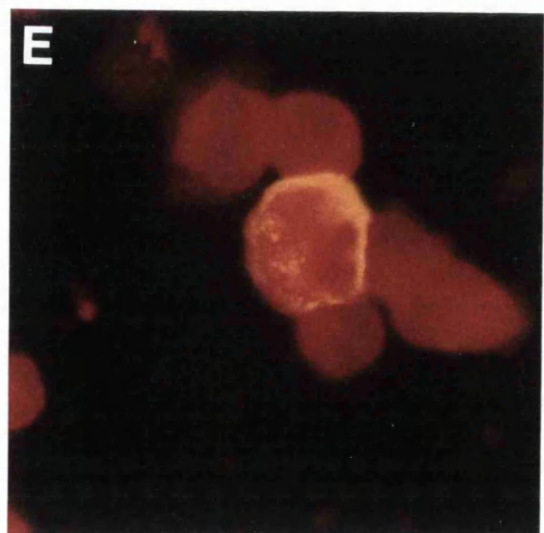
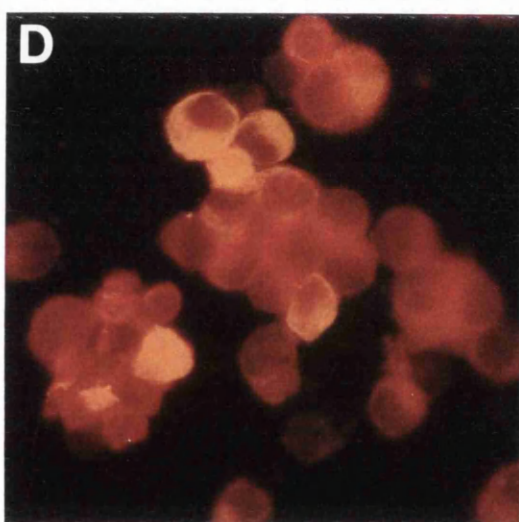
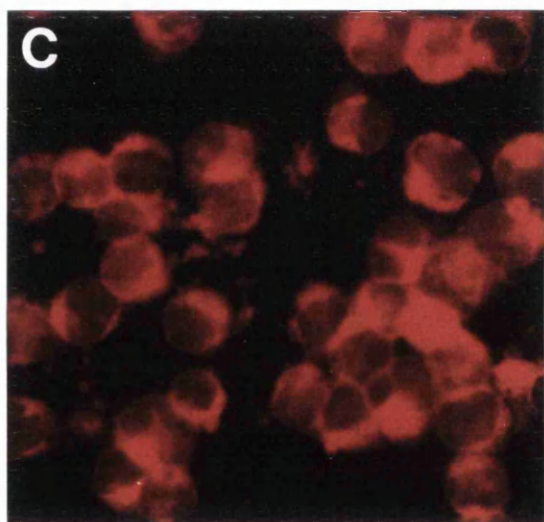
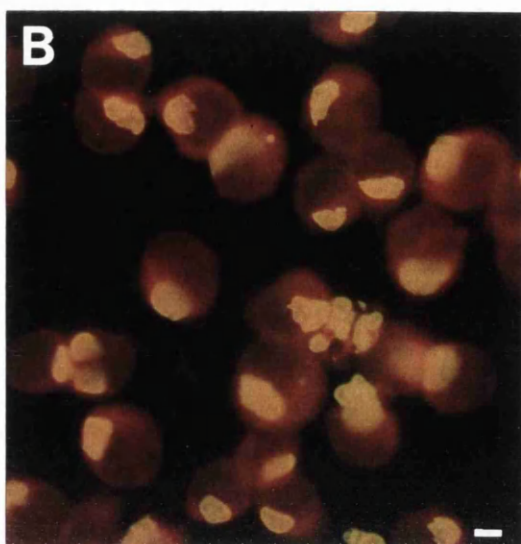
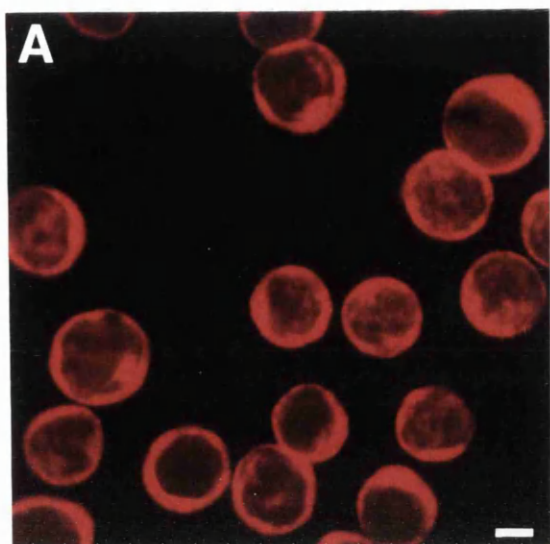
### **2.3.4. Establishment of newly infected cell line**

To determine whether TaHBL20/125 was directly associated with immortalisation by the parasite or was a result of secondary alterations brought about by long-term cell culture, uninfected BL20 cells were infected with *T. annulata* (Hissar) sporozoites using GUTS (2.2.3.) and examined for the presence of TaHBL20/125. Following the initial infection, low serum concentration of either 10% or 5% was used in subsequent passages. BL20 cells proliferate relatively slowly at these low serum levels, whereas infected cells, which have a higher rate of proliferation, are unaffected. Thus, the culture conditions were manipulated to favour the growth of infected cells and establish the new cell line, nTaHBL20. In the early stages the number of infected cells was determined by IFA using the anti-macroschizont monoclonal antibody 1C12 (see 2.2.4.). Figure 2.5.B shows



**Figure 2.5.** Immunofluorescence reactivity of monoclonal antibody 1C12 on fixed slide preparations of (A) uninfected BL20 cells and (B) the newly infected nTaHBL20 cells. Immunofluorescence reactivity of monoclonal antibody 4H5 on fixed slide preparations of; (C) nTaHBL20 cells, passage number 6; (D) nTaHBL20 cells passage number 83; (E) nTaHBL20 cells passage number 83; (F) as E seen under DAPI staining, the presence of a macroschizont is indicated by M.

The magnification bar shown in A also applies to E and F, whereas the magnification bar in B applies to C and D. (Bar = 10  $\mu$ m).

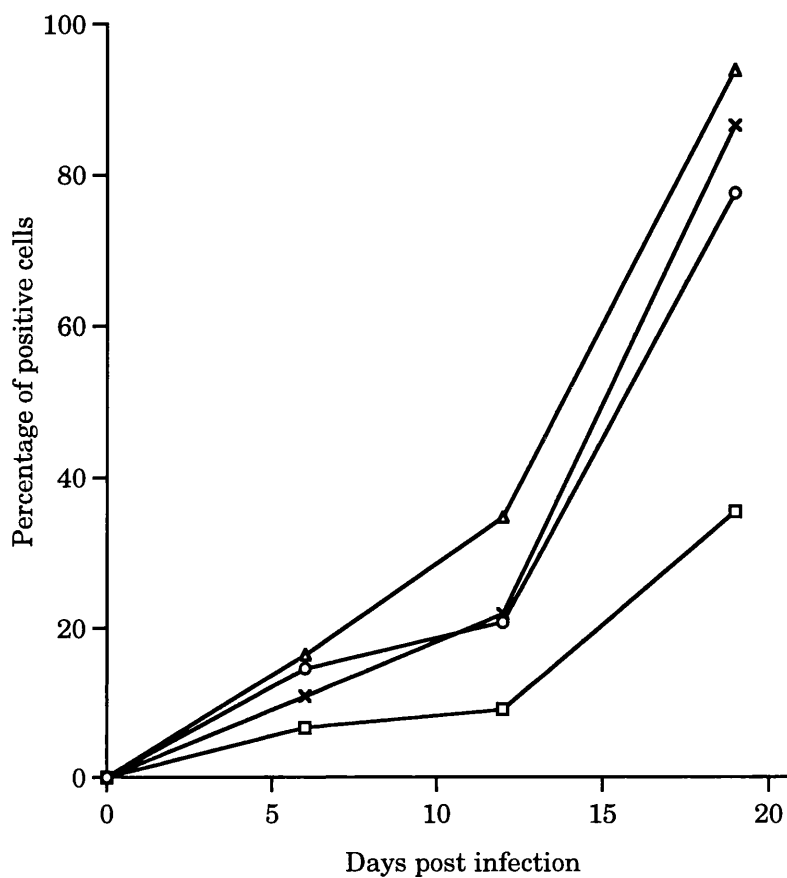


the pattern of reactivity observed with this antibody. Parasite macroschizonts were clearly visible within the cytoplasm of infected cells, allowing an accurate estimation of the number of infected cells to be made. Figure 2.6. shows the rate at which parasitised cell lines were established under a variety of conditions. The highest rate of infection was observed for cells which received the highest tick equivalent (te) of sporozoites (0.4) and were cultured in the lowest serum concentration of 5%. The second highest infection rate was observed where 0.1 te and 5% serum was used. These results suggest that the most important factor in the establishment of the newly infected cell lines was a concentration of serum which favoured proliferation of the infected cells. In three of the cultures a large percentage of parasitised cells were observed (more than 88%) within twenty days of the initial infection. In the passages subsequent to day 20, all cultures eventually attained a high percentage of parasitised cells.

#### **2.3.5. Induction of TaHBL20/125 in nTaHBL20**

The newly infected nTaHBL20 cells were tested for the presence of TaHBL20/125 by IFA and the percentage of cells showing reactivity estimated by counting. At passage 6, no cells were found to react with 4H5 in any of the duplicated nTaHBL20 cultures (Figure 2.5.C). To examine if TaHBL20/125 had perhaps undergone a secondary modification making it unreactive with monoclonal antibody 4H5 by IFA, NP-40 extracts of the nTaHBL20 cultures were immunoblotted, periodate treated and probed with monoclonal antibody 4H5. Comparison was made to similarly treated BL20 and TaHBL20 extracts. As expected BL20 showed no reaction with monoclonal antibody 4H5, and TaHBL20 gave a single specific band of 125 kDa (Figure 2.7.A. lanes 8 and 9 respectively). Monoclonal antibody 4H5 failed to recognise any specific bands in the nTaHBL20 extracts

**Figure 2.6.** Percentage of cells containing macroschizonts in the nTaHBL20 cell lines following infection. Immunofluorescence reactivity of monoclonal antibody 1C12 against nTaHBL20 cells was used to calculate the percentage of infected cells present at different time points following infection.



—○—	(1) 0.4 TE, 10% FCS	—□—	(3) 0.1 TE, 10% FCS
—▲—	(2) 0.4 TE, 5% FCS	—×—	(4) 0.1 TE, 5% FCS

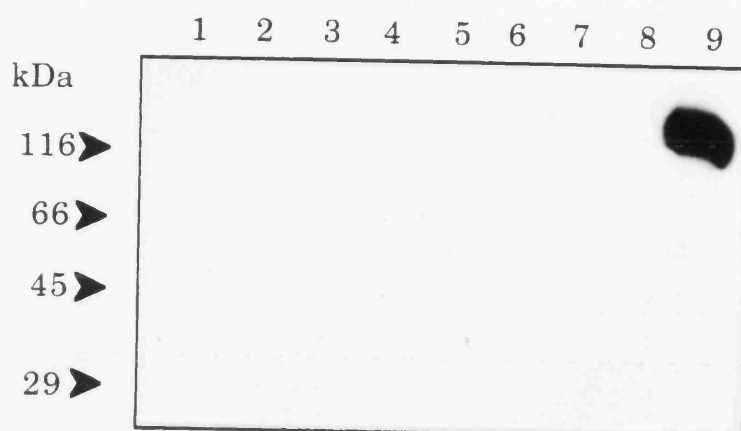
**Figure 2.7.** Analysis of periodate treated immunoblots of newly infected, nTaHBL20 cell extracts probed with monoclonal antibody 4H5 following different lengths of time in culture. (A) extracts made at passage number 8 and (B) extracts made at passage number 83.

Lanes 1 to 7; duplicate nTaHBL20 cultures

Lane 8; BL20

Lane 9; TaHBL20

(A)



(B)



(Figure 2.7.A. lanes 1 to 7), leading to the conclusion that TaHBL20/125 was not present in the nTaHBL20 cell lines at that time.

Duplicate cultures of nTaHBL20 were maintained in culture and periodically tested by both IFA and by immunoblotting for the presence of TaHBL20/125. At passage 83, a small percentage of the cells in all of the duplicate cultures were found to give bright reactivity with 4H5 by IFA (Figure 2.5.D). However, the highest value for the number of brightly positive nTaHBL20 cells was only 7%, compared with over 75% in TaHBL20. Simultaneously staining the IFA slides with DAPI (see 2.2.5.) showed a correlation between cells giving bright reactivity and the presence of macroschizonts (Figure 2.5. E and F). Periodate treated immunoblots of NP-40 extracts from nTaHBL20 cells at passage 83 were probed with monoclonal antibody 4H5. A predominant polypeptide of 125 kDa was seen in nTaHBL20 cultures 1,2, 4 and 5, with a fainter polypeptide of the same size seen in nTaHBL20 cultures 3, 6 and 7 (Figure 2.7.B). This result suggests that TaHBL20/125 had been induced to a varying extent in all of the nTaHBL20 cell lines. By comparing the IFA and immunoblot data, a correlation was found between those cultures showing the highest percentage of positive cells by IFA (Table 2.1.) and those cultures giving the strongest bands by immunoblotting.



**Table 2.1.** Percentage of cells in duplicate nTaHBL20 cultures showing bright positive reactivity with monoclonal antibody 4H5 by IFA during prolonged culture *in vitro*.

Culture	Passage 6	Passage 46	Passage 83
1	0.0	0.5	1.16
2	0.0	0.4	6.95
3	0.0	0.0	0.20
4	0.0	0.2	0.60
5	0.0	0.0	4.44
6	0.0	0.0	0.40
7	0.0	0.0	0.20

## 2.4. Discussion

IFA and immunoblotting using monoclonal antibody 4H5 confirmed the presence of a 125 kDa infection associated molecule in the TaHBL20 cell line. Recognition of this polypeptide by monoclonal antibody 4H5 on immunoblots was observed only when non-denaturing conditions were used in the electrophoresis. The inclusion of 2-mercaptoethanol in normal sample buffer and the heating of samples to 100°C is generally used to disrupt the physical structure of proteins and allow polypeptides to migrate during SDS-PAGE electrophoresis as a function of molecular mass. Under these conditions monoclonal antibody 4H5 failed to recognise any molecule in TaHBL20. From this it was possible to conclude that the antibody recognised a conformational epitope, and that physical disruption of the structure of TaHBL20/125 resulted in a loss of reactivity by the antibody. Previous indirect evidence had characterised the 4H5 antigen as a glycoprotein (Shiels, *et al.*, 1989). This was confirmed directly by immunoprecipitation of the 125 kDa antigen from TaHBL20 extracts with monoclonal antibody 4H5 and subsequent detection on immunoblots by the anti-glycoprotein polyclonal antibody.

The experiments represented in Figure 2.3. indicated that there were variations in the ability of monoclonal antibody 4H5 to detect TaHBL20/125. When immunoblots of TaHBL20 were treated with periodate, an increase in signal was observed for the TaHBL20/125 antigen (Figure 2.3. lanes 2 and 4). A possible explanation for this increase was that the epitope for monoclonal antibody 4H5 was, to some extent masked by oligosaccharide residues. Sodium periodate oxidises hydroxyl groups on the hexose ring of carbohydrates, thus breaking the ring and disrupting the physical integrity of any oligosaccharides containing this structure (Eylar

and Jeanloz, 1962). Disruption of carbohydrate residues could have increased the access of the antibody to its epitope, resulting in the observed increase in signal.

Previously monoclonal antibody 4H5 had failed to detect any polypeptide in uninfected PBLs or in the uninfected cell line BL20. Experiments were carried out to investigate the induction kinetics of TaHBL20/125 following infection of BL20 by *T. annulata* (Hissar) sporozoites. This experiment essentially re-created the TaHBL20 cell line. Shortly after the infection of BL20 (passage 6) cells were assayed by IFA with monoclonal antibody 4H5 for the presence TaHBL20/125, however, no reactivity was observed. Recognition of TaHBL20/125 by monoclonal antibody 4H5 was known to be sensitive to conditions which affect the structural integrity of polypeptides. This antigen was also known to show variability between different cell lines (Shiels, *et al.*, 1989). Therefore, it was possible that in the new cell lines, TaHBL20/125 could have undergone structural modifications which resulted in a lack of recognition by monoclonal antibody 4H5 by IFA. Periodate treated immunoblots of the nTaHBL20 cell lines were probed with monoclonal antibody 4H5, however, no polypeptide was detected under these conditions. By the criteria previously established for the detection of TaHBL20/125, the molecule was considered to be absent in the nTaHBL20 cell lines at this time, although it was possible that the apparent absence of the antigen could have been the result of still further structural alterations to the 4H5 epitope which affected recognition by the antibody.

Prolonged culture *in vitro* is known to alter the phenotype of cells. For example, the activities of protease enzymes has been shown to decrease following long term cell culture (Baylis, *et al.*, 1992). To investigate if prolonged culture *in vitro* would affect the expression of TaHBL20/125 by

nTaHBL20, the cells were maintained and periodically re-tested for the presence of TaHBL20/125. A noticeable change in the phenotype of the nTaHBL20 cell lines occurred at around passage 83. When tested for reactivity with 4H5 by IFA a few cells, between 0.2% and 7%, in some of the cultures showed intense reactivity (Figure 2.5.D.). Similarly, when periodate treated immunoblots were probed with 4H5, a polypeptide of 125 kDa was observed in the cell lines (Figure 2.7.B.). Thus, it would seem that prolonged culture *in vitro* had induced the expression of TaHBL20/125, albeit at low level. Different duplicate cultures were found to have varying levels of TaHBL20/125. These variations in expression could have arisen as a result of subtle differences in the culture conditions found in each flask over time. Alternatively, the variations could have been a result of mixed sporozoite infections as, although derived from identical host cells, it is likely that the GUTS extract contained mixed parasite populations.

Several changes associated with the transformation process have been described previously (see 1.7.), and host molecules which are up-regulated by the parasite have been found to be variably expressed in a similar way to the expression patterns observed for TaHBL20/125 in nTaHBL20 cells. For example, in cells infected with *T. parva*, the percentage of cells positive for the expression of the Tac antigen component of the IL-2 receptor varies between different cell lines and between experiments. These fluctuations have been found in both cloned and uncloned cell lines and were also observed for other surface differentiation antigens (Dobbelaere, *et al.*, 1990). Similarly, the expression pattern of the Tac antigen varied in *T. annulata* infected cells, where the percentage of positive cells ranged from 94% to 6% in different cell lines. Therefore, the low level expression of TaHBL20/125 in nTaHBL20 compared to TaHBL20 cells does not necessarily preclude the conclusion that its expression is associated with infection by the parasite.

A similar experiment to the one described above was carried out by Leila Ben Miled (unpublished data) and the results are presented in Table 2.2. PBLs isolated from 6 different cows were infected with sporozoites of *T. annulata* (Hissar) and tested for reactivity with monoclonal antibody 4H5 by fluorescent assay cell sorting (FACS) analysis. At eight days post infection, all 4 cultures showed a high percentage of cells with positive reactivity for monoclonal antibody 4H5, (59% to 88%). By day 14 two of the cultures, from the A70 and A435 cows, were found to contain over 98% positive cells. As the substantial increase in the percentage of cells showing positive reactivity from day 0 to day 14 was probably a reflection of the increasing proportion of infected cells in the PBL cultures, the results indicate that in PBLs, induction of the antigen recognised by monoclonal antibody 4H5 was brought about soon after immortalisation was initiated. Thus, two sets of conflicting data on the kinetics of 4H5 antigen expression have been obtained

An explanation for this discrepancy could be that there is a difference between the nature of the host cells infected in each experiment. BL20 is a lymphoblastoid cell line, probably of B-cell origin, originally isolated from a case of sporadic bovine leukosis (Olobo and Black, 1989). As this cell line was already transformed, it is not clear how this might affect the subsequent alterations to host cell gene expression brought about by the parasite. Following infection by *T. annulata*, the BL20 cells increased their rate of proliferation and formed large clumps of cells characteristic of the original TaHBL20. Alterations associated with the parasite had clearly taken place. However, some of the changes in gene expression associated with parasite transformation of normal PBLs might be more difficult to bring about in an immortalised cell where changes to gene expression have

**Table 2.2.** Percentage of peripheral blood lymphocytes (PBLs) showing positive reactivity for monoclonal antibody 4H5 by fluorescent antibody cell sorting (FACS) analysis over a 14 day period from four different cows infected with *T. annulata* (Hissar) sporozoites.

Cow	Day 0	Day 2	Day 4	Day 8	Day 11	Day 14
A65	0	6.33	26.49	82.44	----	----
A434	0	19.58	12.20	58.96	----	----
A70	0	----	13.59	88.50	97.16	98.65
A435	0	----	11.0	72.74	90.38	98.50

already occurred. It is impossible to establish if similar patterns of TaHBL20/125 expression were displayed during the establishment of the original TaHBL20 cell line, as early passage numbers do not exist. Only prolonged culture *in vitro* will determine if TaHBL20/125 expression shown by the nTaHBL20 cell lines will achieve the levels observed for the original TaHBL20. Taken together the results imply an association of elevation of 4H5 antigen expression, but it remains unclear whether this event is a primary or secondary response to infection of the host cell by the parasite.

By examining the characteristics of TaHBL20/125, it is possible to find similarities with other molecules in uninfected cells of a related lineage and provide possible clues as to its identity. The most interesting feature of the 4H5 antigen is its variable molecular mass between cell lines. Many of the surface molecules identified on the surface of human leukocytes display similar variations in molecular mass and these variations have been found to be a result of essentially three different mechanisms.

The carcinoembryonic antigen (CEA) is a prototype family of highly homologous glycoproteins which have been reported to have a variety of functions including cell adhesion (Kuijpers, *et al.*, 1992), signal transduction (reviewed in Stanners, *et al.* 1995), collagen binding (reviewed in Thompson, *et al.*, 1991), ecto-ATPase activity (reviewed in Thompson, *et al.*, 1991), and bile acid transport (Sippel, *et al.*, 1993). Individual members of the CEA have traditionally been hard to characterise due to the difficulty in obtaining monoclonal antibodies which do not cross react with other members of the family (Engvall, *et al.*, 1978; Paxton, *et al.*, 1987; Shively and Beatty, 1985; Thompson, *et al.*, 1987). It is possible that monoclonal antibody 4H5 could be recognising a shared epitope present on different, but possibly related molecules, in the different infected cell lines. When

leukocytes become immortalised by the parasite they stop expressing a number of cell surface markers, making it difficult to determine precisely the type of cell infected (Glass and Spooner, 1990; Spooner, *et al.*, 1989; Spooner, *et al.*, 1988). The possibility exists that if the parasite brings about transformation of distinct cell types the induction of different, but related molecules could take place.

A second way by which variable molecular mass might be brought about is by post-transcriptional modification of gene expression for example, variable intron splicing of mRNA molecules. The occurrence of a number of alternatively spliced mRNA appears to be a typical feature of cell adhesion molecules (CAMs). Neural-CAM (NCAM) has at least twenty seven alternatively spliced forms expressed during rat heart development (Reyes, *et al.*, 1991). Therefore, the variation in molecular mass of the 4H5 antigen could be brought about by the translation of different splice variants of the same gene in different cell lines.

The third and most likely explanation for the variable molecular mass, is that of post-translational modification by, for example, variable glycosylation. This type of variation also been seen in the surface glycoproteins of leukocytes. Leukosialin (CD43), a major sialoglycoprotein, has been shown to have a variable molecular mass of between 95- and 150 kDa due to differences in the O-linked glycan attached to the molecule (Carlsson and Fukuda, 1986a). The structure of the O-linked glycan is characteristic of cell lineage (Carlsson, *et al.*, 1986b) and different stages of differentiation (Fukuda, *et al.*, 1986). Thus, the gene encoding the antigen recognised by monoclonal antibody 4H5 could undergo differential post-translational modifications which are characteristic of a particular cell type.



Little is known about the true identity of TaHBL20/125 and a number of important questions remain unresolved. Although assumed to be of host origin, no direct evidence exists as to the identity, function or mechanism of up-regulation of this antigen in *T. annulata* infected cells. Similarly the mechanism of variation in molecular mass displayed by this molecule is also unknown. A number of these questions would be most easily addressed by the isolation of the gene encoding the 4H5 antigen, and this was a subsequent aim of this study.

## **Chapter 3**

# Characterisation of an antigen recognised by monoclonal antibody 4H5 in HL-60

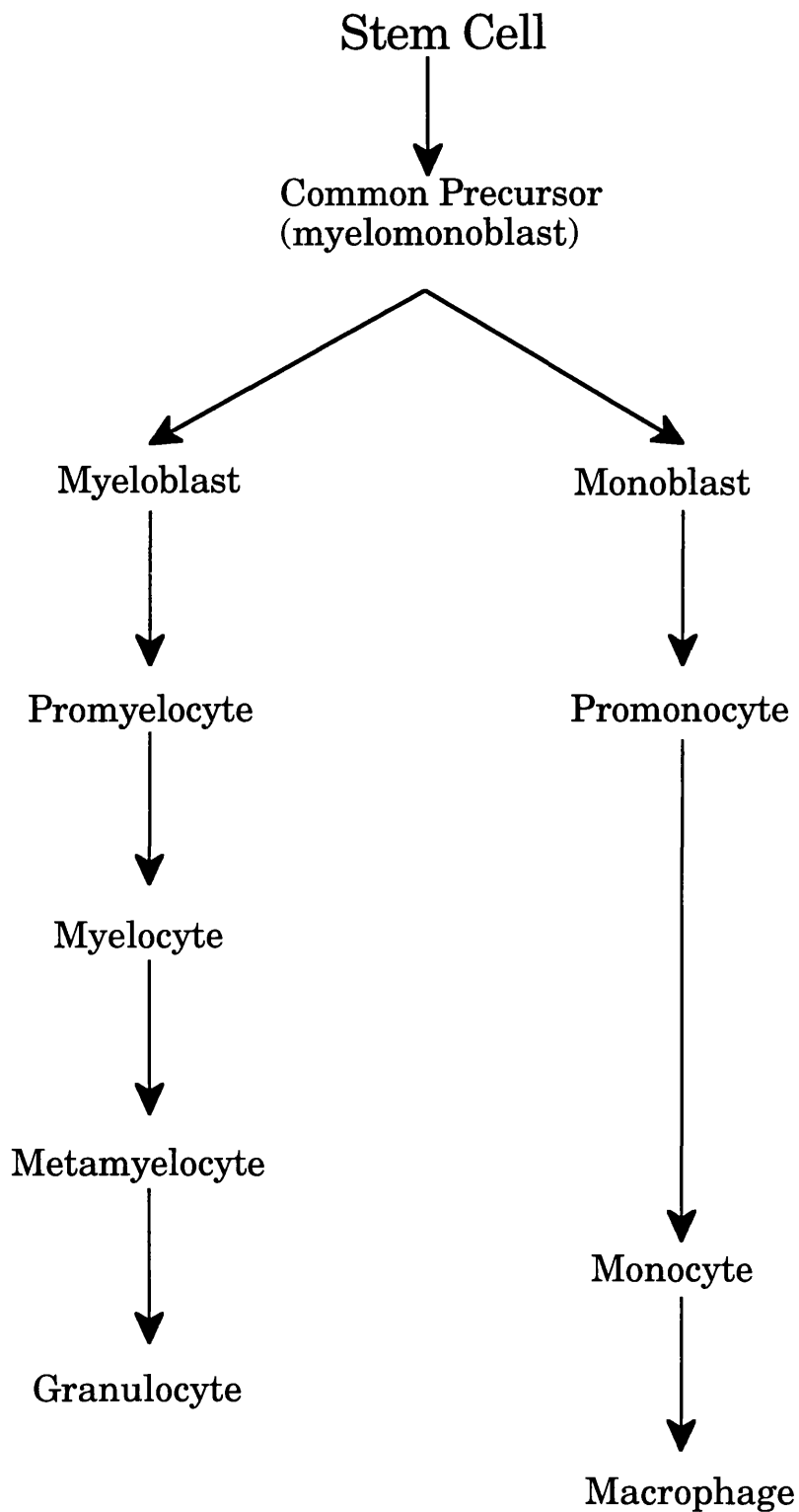
## 3.1. Introduction

The preceding chapter described the characterisation of a molecule of probable host cell origin, which is up-regulated by the presence of the parasite within the host cell. *T. annulata* infects cells of the bovine immune system which are positive for MHC class II antigens, namely B cells and monocytes/macrophages (Glass, *et al.*, 1989; Spooner, *et al.*, 1989; Spooner, *et al.*, 1988). Up-regulation of host molecules as a result of parasite induced immortalisation is likely to occur via aberrant expression of genes associated with cells of these developmental lineages. Therefore, it was of interest to investigate if any parallels exist between altered antigenic expression in *Theileria* infected cells and in uninfected cells of a related lineage. The reactivity of monoclonal antibody 4H5 had been previously tested against normal and mitogenically stimulated PBLs and found to be negative (Shiels, *et al.*, 1986b), it was decided therefore that the reactivity should be tested against other cells lines of the monocyte/macrophage line. One of the most extensively studied cell lines with similarities to the lineage of *T. annulata* infected cells is the human leukemic cell line HL-60.

The HL-60 cell line was originally isolated from a patient with acute myeloid leukaemia (Collins, *et al.*, 1977). Unlike other cells isolated from patients with similar leukemias, where growth *in vitro* is often limited to a few cell divisions due to a lack of patient derived exogenous growth factors, HL-60 displays factor independent continuous proliferation in culture. The characteristic which has attracted most interest in this cell line however, is its capacity to differentiate *in vitro* into a variety of different cell types of

the myelomonocytic cell lineage, reviewed in Collins (1987). HL-60 can be induced to differentiate into granulocytes, monocytes and macrophage-like cells by a variety of agents. When considering this cell line it is important to note that these designations are somewhat arbitrary as certain inducers give rise to cells with characteristics overlapping these categories, and when compared to the normal cell counterpart, differentiated HL-60 cells may lack certain lineage specific characteristics. The bipotency of HL-60 also causes some difficulty in the assignment of the cell line to an appropriate place in the hierarchy of the granulocyte and monocyte/macrophage cell lineage (Figure 3.1). It is possible that this is not an abnormal cellular state brought about by transformation and *in vitro* culture, and that the determined hierarchy represented in Figure 3.1. may not be absolutely correct (Fibach, *et al.*, 1982).

HL-60 cells are predominantly promyelocytes but, in culture, spontaneous differentiation to mature myelocytes, metamyelocytes and granulocytes occurs in approximately 5% of cells. The spontaneous differentiation of HL-60 to granulocytes can be increased by a variety of agents which include polar-planar compounds such as DMSO (Collins, *et al.*, 1978) and a diverse range of other agents such as, retinoic acid (Breitman, *et al.*, 1980), actinomycin D (Collins, *et al.*, 1980), tunicamycin (Nakayasu, *et al.*, 1980), hypoxanthine (Nakayasu, *et al.*, 1980) and dibutyl cAMP (dbcAMP) (Sirak, *et al.*, 1990). Differentiation towards granulocytes is somewhat defective, in that most cultures consist of predominantly metamyelocytes; these cells lack lactoferrin (Newburger, *et al.*, 1979; Olsson and Olofsson, 1981), and lactate dehydrogenase (LDH) isoenzyme profiles differ quantitatively from normal granulocytes (Pantazis, *et al.*, 1981). The mechanism by which the various inducers bring about granulocyte differentiation is unknown, but induction by most of these agents results in terminal differentiation, that is



**Figure 3.1.** Granulocyte and monocyte/macrophage cell lineages. Adapted from Birnie (1988).

cells are no longer capable of proliferation. A minimum of 12 hours exposure to DMSO or retinoic acid is necessary before any differentiation takes place, with maximum differentiation usually requiring constant exposure (Breitman, *et al.*, 1980; Tarella, *et al.*, 1982).

Like granulocytes, differentiation towards monocytes can be induced by a variety of compounds such as vitamin D<sub>3</sub> (Miyaura, *et al.*, 1981), sodium butyrate (Boyd and Metcalf, 1984), TNF (Trinchieri, *et al.*, 1986) and IFN  $\gamma$  (Ball, *et al.*, 1984). Recently it has been shown that the changes in myeloid cell surface antigens induced by vitamin D<sub>3</sub> parallel the expression pattern of these molecules in normal monocytes (Brackman, *et al.*, 1995). Monocytes are precursors in the cell lineage and unlike differentiation towards granulocytes or macrophages, monocytic differentiation is not terminal and can be accompanied by cell proliferation (Ball, *et al.*, 1984; Hemmi and Breitman, 1987).

In contrast to granulocytic and monocytic differentiation, the formation of macrophages is induced by significantly fewer compounds, with phorbol esters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) being most commonly used (Rovera, *et al.*, 1979). HL-60 cells induced to differentiate to macrophages are easily distinguished from monocytic cells by their intense adherence to plastic in culture. In addition, they do not display specific surface antigens present on normal and HL-60 monocytes (Ferrero, *et al.*, 1983; Graziano, *et al.*, 1983; Todd, *et al.*, 1981) and do not reduce NBT (Newburger, *et al.*, 1981; Skubitz, *et al.*, 1982). TPA induction of HL-60 occurs rapidly, with significant differentiation to macrophages occurring after only 20 minutes exposure (Rovera, *et al.*, 1979). TPA induced differentiation occurs in the absence of cell division or DNA synthesis and is associated with a rapid loss of proliferative capacity.

The inherent characteristics of the HL-60 cell line made it a good candidate for the further investigation of parallels between altered antigenic expression following *T. annulata* infection and similar alterations in related cell lineages. The ability of HL-60 to undergo differentiation to a number of different but related cell types allowed examination of the surface antigens of granulocytes and monocytes/macrophages for reactivity with monoclonal antibody 4H5.

### **3.1.1. Aims**

The primary aim of work presented in this chapter was to investigate if an antigen recognised by monoclonal antibody 4H5 was present in uninfected cells of a related cell lineage. Initially cells of the human promyelocytic leukemia cell line HL-60 were tested for reactivity with the antibody. Any polypeptides recognised by 4H5 in HL-60 cells would then be characterised with a view to further identification. Changes in gene expression are known to accompany differentiation. The third aim of this chapter was to further investigate if changes in the expression of a putative 4H5 antigen in HL-60 was altered following differentiation to a variety of different cell types.

## **3.2. Material and Methods**

### **3.2.1. Cell Lines and Culture Conditions**

HL-60 cells were maintained in culture at 37°C in RPMI-1640 (Gibco) supplemented with 10% heat inactivated foetal calf serum, 8 mg ml<sup>-1</sup> streptomycin, 8 units ml<sup>-1</sup> penicillin, 0.6 mg ml<sup>-1</sup> amphotericin B and 0.05% NaHCO<sub>3</sub>. Cells were induced to differentiate along the granulocyte pathway by incubation in fresh medium at 4 x 10<sup>5</sup> cells ml<sup>-1</sup> in the presence of either 1.3% (v/v) DMSO or 10<sup>-7</sup> M dbcAMP (Sigma). Differentiation along the monocytic pathway was achieved by incubation with phorbol 12 myristate-1,3-acetate (PMA, Sigma) or by incubation in 1.3% DMSO for 5 days, followed by 3 washes with pre-warmed RPMI-1640 and resuspension in medium containing 20 ng ml<sup>-1</sup> PMA in DMSO (Nguyen, *et al.*, 1993). PMA was dissolved as a stock in DMSO. 2 µl of stock was added to give a final concentration of 20 ng ml<sup>-1</sup> PMA. Control cultures contained a similar volume of DMSO.

### **3.2.2. Antisera**

Monoclonal antibody 4H5 was as described in 2.2.4. Monoclonal antibody 1D11 was raised against piroplasm infected erythrocytes and was used as undiluted hybridoma supernatant (Glascodine, *et al.*, 1990). The anti-human β<sub>2</sub>-microglobulin serum (Sigma) was diluted 1:200 in block buffer (see 2.2.8).

### **3.2.3. NBT Reduction Test**

1 ml of cell suspension was incubated for 20 minutes at 37°C with an equal volume of 0.2% nitro blue tetrazolium (NBT, Sigma) in PBS, and 200 ng of PMA, (Segal, 1974). Following incubation, 100 µl aliquots of cell suspension were centrifuged at 240 g for 5 minutes onto glass slides in a Shandon



cytopsin 2 apparatus. Cells were visualised under oil at x 1 000 magnification, using a Leitz Wetzlar SM-Lux light microscope. Scores of NBT positive cells were performed on a minimum of 500 cells.

### **3.3. Results**

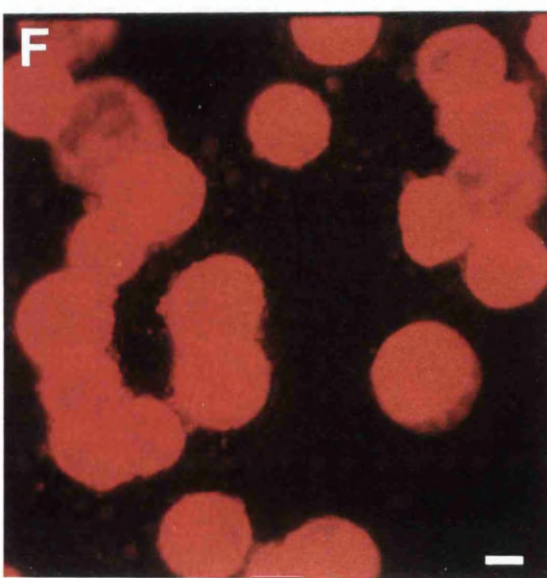
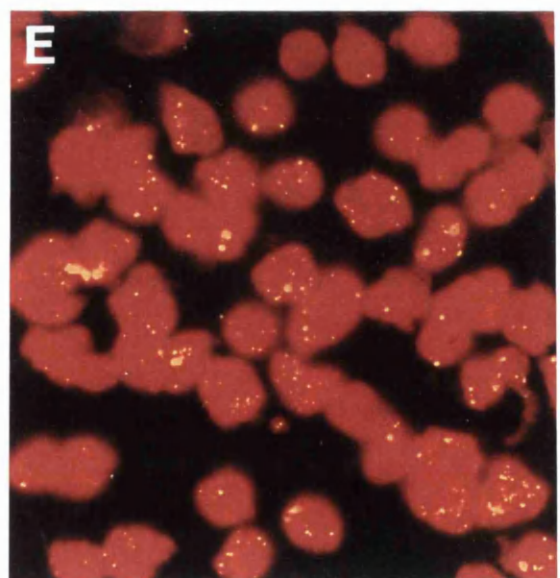
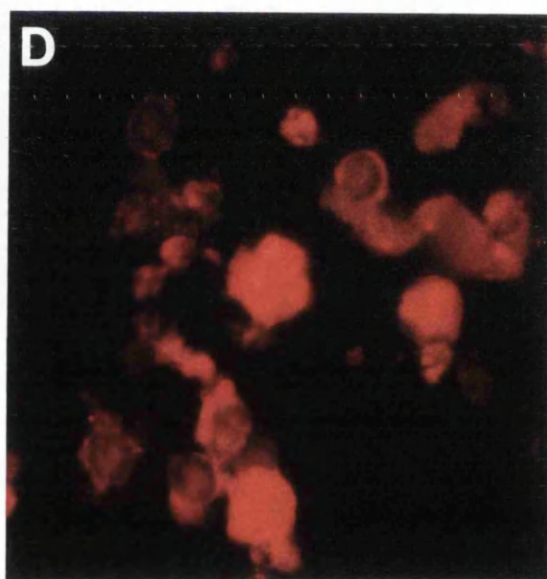
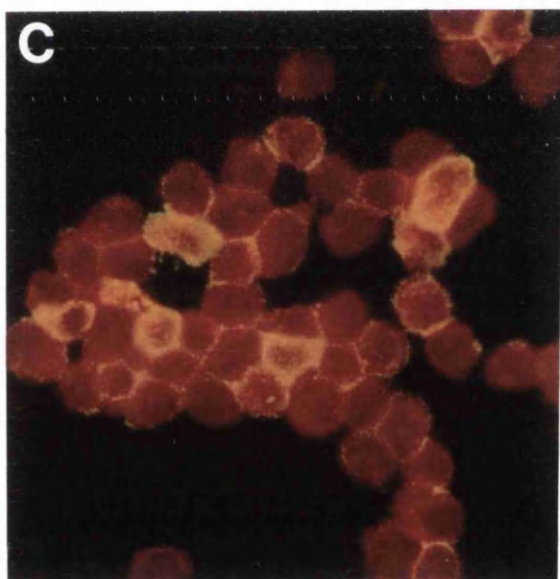
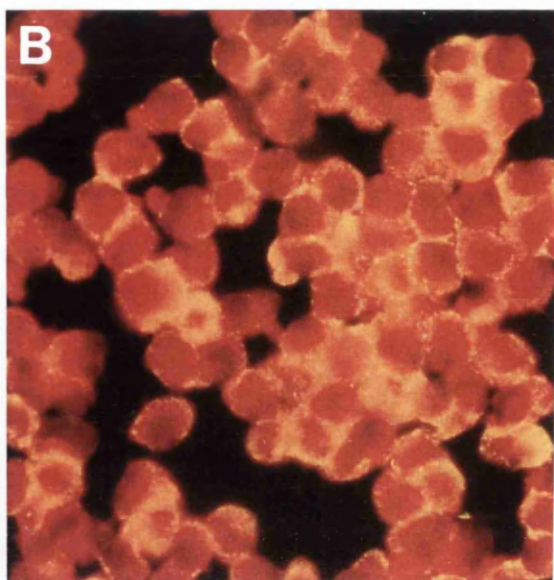
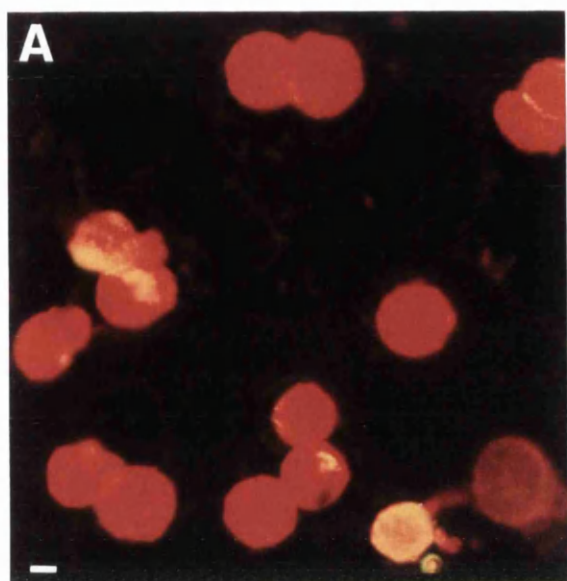
#### **3.3.1. Detection of antigen recognised by monoclonal antibody 4H5 on HL-60 cells during differentiation towards granulocytes**

When monoclonal antibody 4H5 was reacted against HL-60, the majority of cells were negative but low levels of staining were observed for a significant number of cells, and a few cells showed strong reactivity (Figure 3.2.A). To test if reactivity could be altered following differentiation, cultures were differentiated towards granulocytes by incubation with either DMSO (1.3%) or  $10^{-7}$  M dbcAMP, or towards macrophages by incubation with 20 ng ml<sup>-1</sup> PMA, and the number of cells showing positive reactivity counted. A substantial alteration in the reactivity of 4H5 was induced by this treatment, the number of cells characterised by strong reactivity increased in the granulocyte cultures (Figure 3.2.B and C). When monoclonal antibody 4H5 was used in a surface IFA on HL-60 granulocytes, a speckled pattern of reactivity was observed (Figure 3.2.E) indicating that the antigen recognised by 4H5 had a surface location. In all culture conditions tested no reactivity was detected on HL-60 with the parasite specific control monoclonal antibody 1D11 (Figure 3.2.F).

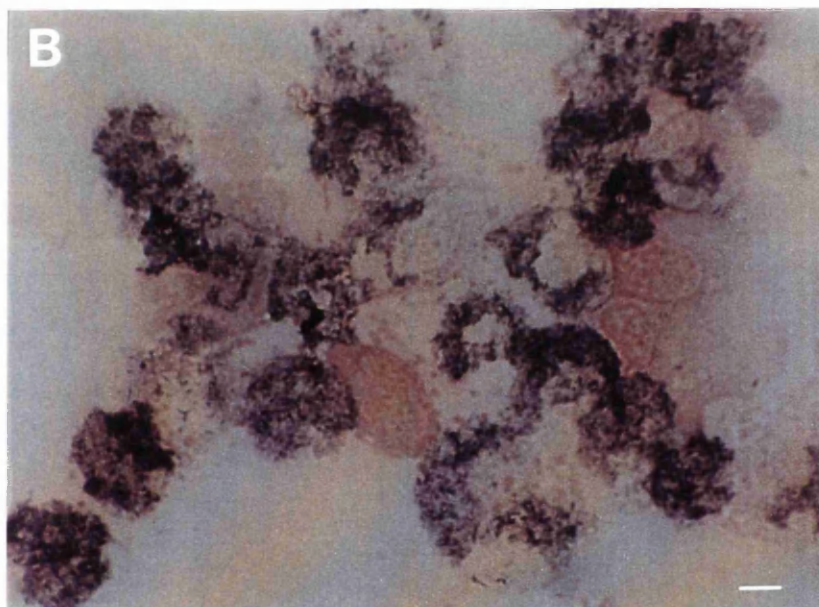
Differentiation of HL-60 cells towards granulocytes was confirmed by NBT assay. TPA induces respiratory burst activity in HL-60 cells induced to differentiate towards granulocytes, resulting in the generation of superoxide anion and the reduction of soluble NBT to blue-black insoluble formazan. Before DMSO induced differentiation, HL-60 cells were negative for the NBT assay (Figure 3.3.A). Following DMSO induced differentiation to granulocytes, a large number of NBT positive cells were observed (Figure 3.3.B). Differentiation towards macrophages was confirmed by observed

**Figure 3.2.** Immunofluorescence reactivity of monoclonal antibody 4H5 on fixed slide preparations of; (A) the human leukaemia cell line HL-60; (B) HL-60 induced to differentiate towards granulocyte like cells by a continuous 5 day exposure to 1.3% DMSO; (C) HL-60 induced to differentiate towards granulocyte like cells by a continuous 5 day exposure to  $10^{-7}$  M dbcAMP; (D) HL-60 induced to differentiate towards macrophage like cells by 24 hour incubation with 20 ng ml<sup>-1</sup> PMA; (E) surface immunofluorescence reactivity of monoclonal antibody 4H5 on DMSO induced HL-60 cells; (F) immunofluorescence reactivity of monoclonal antibody 1D11 on fixed slide preparations of DMSO induced HL-60 cells.

The magnification bar shown in A also applies to B to E. (Bar = 10  $\mu$ m).



**Figure 3.3.** NBT assays of cytopsin preparations of HL-60 cells; (A) HL-60 cells; (B) HL-60 induced to differentiate towards granulocyte like cells by a continuous six day exposure to 1.3% DMSO. (Bar = 10  $\mu\text{m}$ )



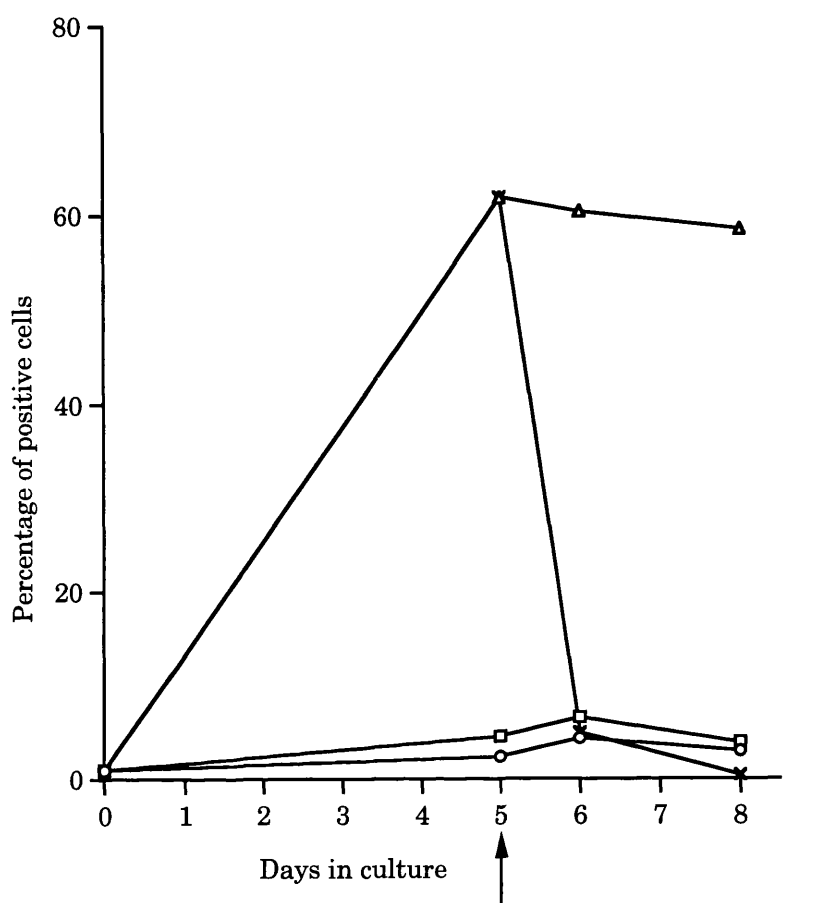
changes in cell morphology, as cells rapidly became intensely adherent to the surface of the culture flask (data not shown).

### **3.3.2. Restriction of the 4H5 antigen to cells of the granulocyte lineage**

To determine if the antigen recognised by monoclonal antibody 4H5 in HL-60 was restricted to granulocytes, cells were induced to differentiate towards macrophages using 20 ng/ml PMA. Comparison of IFA reactivity on these cells (Figure 3.2.D) with DMSO induced granulocytes (Figure 3.2.B) showed that elevated reactivity of 4H5 was not observed in PMA induced cultures. To examine whether the elevated reactivity of granulocytes could be reversed by inducing differentiation towards macrophage like cells, the percentage of 4H5 positive cells was assessed daily during continuous exposure of HL-60 cells to 1.3% DMSO for 5 days, followed by replacement of DMSO with PMA for 3 days. The profile of reactivity was compared to a control and to cultures incubated continuously in DMSO or PMA. The results represented by Figure 3.4. show that continuous exposure to DMSO induced a rise in the number of cells showing positive fluorescence, while cells continuously exposed to PMA, which were morphologically identified as macrophages, had levels of reactivity similar to control cultures. Replacement of DMSO with PMA on day 5 resulted in the rapid loss of expression of the 4H5 antigen and appearance of macrophage type cells within 24 hours. By day 8 the proportion of positive cells in these cultures had fallen to 0.4 %. Thus, it appears that detection of the 4H5 antigen at elevated levels is restricted to cells induced to differentiate towards granulocytes.

**Figure 3.4.** Pattern of fluorescent reactivity shown by HL-60 with monoclonal antibody 4H5 over an 8 day period under a variety of conditions. The percentage of cells showing intense reactivity with the antibody were recorded as follows; (1) control cultures containing  $2 \times 10^{-3}\%$  DMSO; (2) induction of differentiation towards granulocytes by continuous exposure to 1.3% DMSO; (3) induction of differentiation towards macrophages by continuous exposure to  $20 \text{ ng ml}^{-1}$  PMA; (4) induction of differentiation towards granulocytes for 5 days followed by the removal of DMSO and differentiation towards macrophages by incubation with PMA, time of replacement is indicated with an arrow.





- (1) control culture
- △— (2) granulocyte culture
- (3) macrophage culture
- ×— (4) granulocyte/macrophage culture

### 3.3.3. Identification of g160 in HL-60 by immunoblotting

Previously, monoclonal antibody 4H5 was shown to detect a 125 kDa antigen in *T. annulata* infected cells by immunoblotting and periodate treatment under non-reducing conditions (See 2.3.2.). To characterise the 4H5 antigen at the molecular level in HL-60, NP-40 extracts were prepared from normal HL-60 cells and cells induced to differentiate into granulocytes by a five day incubation in 1.3% DMSO. In order to obtain specific detection of the antigen by immunoblotting, electrophoresis had to be performed under non-reducing conditions and the Western blots treated with periodate (See 2.2.8. and 2.2.9.). This protocol identified a molecule with an approximate molecular mass of 160 kDa in both normal HL-60 cells (Figure 3.5. lane 1) and DMSO induced granulocytes (Figure 3.5. lane 2), with the granulocyte extract showing a greater level of band intensity. Normal SDS-PAGE conditions failed to specifically detect any antigen in HL-60 cells (Figure 3.5. lanes 3 to 4). In contrast to *Theileria* infected cells (Figure 3.6. lanes 1 and 3) detection of the g160 kDa polypeptide under non reducing conditions was found to be totally dependant on periodation, as detection of the g160 kDa antigen was only observed following periodate treatment (Figure 3.6. lanes 2 and 4).

In a second experiment, NP-40 extracts were made at daily intervals over a 5 day period following the addition of DMSO, and analysed by immunoblotting. This experiment showed that the level of the g160 kDa antigen increased as the time course of differentiation progressed (Figure 3.7.A). Analysis of the same samples with antiserum raised against  $\beta_2$ -microglobulin showed constitutive levels of this polypeptide throughout the time course (Figure 3.7.B). This result indicated that the observed elevation of the g160 kDa antigen was due to an increase in production associated with the differentiation process.

**Figure 3.5.** Analysis of HL-60 NP-40 extracts on immunoblots probed with monoclonal antibody 4H5 following non-denaturing and normal SDS-PAGE conditions, and periodate treatment.

Lane 1; HL-60

Lane 2; HL-60 induced to differentiate towards granulocytes by a 5 day continuous exposure to 1.3% DMSO

Lanes 3 and 4; as lanes 1 and 2 immunoblotted under normal SDS-PAGE conditions.

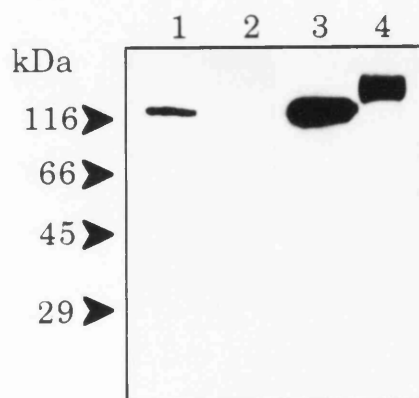
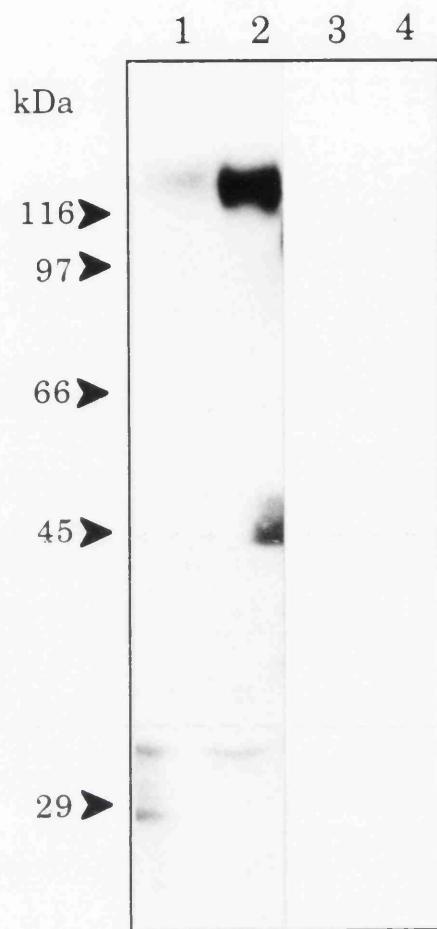
**Figure 3.6.** Analysis of HL-60 extracts on immunoblots probed with monoclonal antibody 4H5 following non-denaturing SDS-PAGE. NP-40 cell extracts were made from HL-60 granulocytes induced to differentiate by a 5 day incubation with 1.3% DMSO and immunoblotted with and without periodate treatment.

Lane 1; TaHBL20, positive control

Lane 2; HL-60 granulocytes

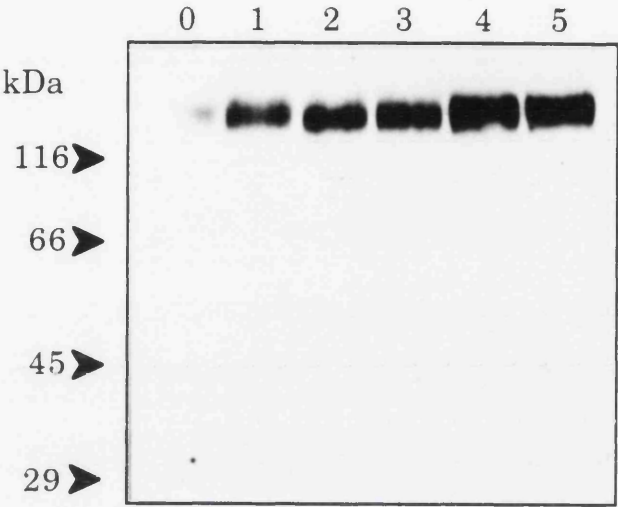
Lane 3; TaHBL20, treated with periodate

Lane 4; HL-60 granulocytes, treated with periodate

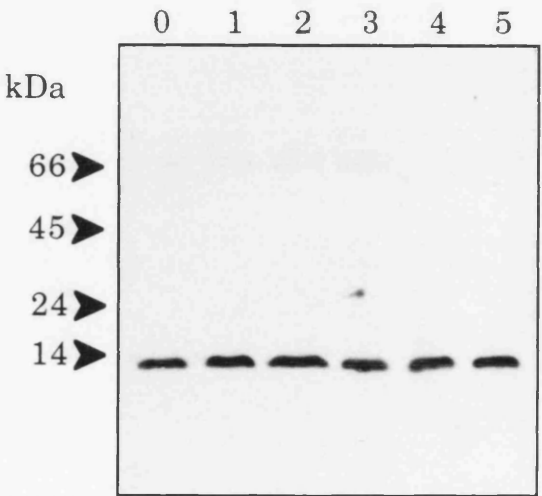


**Figure 3.7.** Analysis of HL-60 extracts on immunoblots during a 5 day DMSO induced differentiation time course. NP-40 extracts were made every day for 5 days from HL-60 cells incubated in the presence of 1.3% DMSO and immunoblotted using; (A) monoclonal antibody 4H5 or (B) anti-human  $\beta_2$ -microglobulin antibody. Lanes 0 to 5 refer to the day on which each extract was made.

(A)



(B)



### 3.4. Discussion

Infection and immortalisation of bovine leukocytes by *T. annulata* is known to be associated with a number of molecular and phenotypic alterations to the host cell (see 1.7.). As changes to gene expression result from immortalisation and differentiation of eukaryotic cells in general, it was investigated whether alterations associated with *T. annulata* infection could be observed in uninfected cells of a related lineage.

The results showed that monoclonal antibody 4H5 detected an antigen in HL-60 cells, the reactivity of which was increased following DMSO or dbcAMP induced differentiation towards granulocytes (Figure 3.2.). This increase in reactivity was specific to cells undergoing granulocyte formation, as no similar increase in reactivity was observed on cells induced towards macrophage differentiation. Confirmation of the lineage specificity of this molecule was provided when it was found that high level reactivity of the antibody was rapidly down-regulated by the replacement of DMSO with PMA, and which coincided with conversion of granulocytes to macrophages (Figure 3.4.). Similar results have been observed previously, as simultaneous treatment with both inducers resulted in macrophage differentiation and, following replacement of DMSO by PMA, cells were found to switch from granulocyte to macrophage differentiation (Liebermann, *et al.*, 1981). Nguyen, *et al.* (1993) showed that the zinc finger transcription factor, *Egr-1*, was essential for and restricted differentiation of HL-60 cells towards macrophages. DMSO induced HL-60 cells did not express *Egr-1*, however removal of DMSO after 5 days, followed by replacement with PMA resulted in macrophage differentiation and the transcriptional activation of *Egr-1* within 1 hour of replacement. It is likely that, in addition to the rapid induction of macrophage specific genes

following PMA stimulation of granulocytes, rapid down regulation of granulocyte specific genes also accompanies this change. However, it is not yet known whether Erg-1 is directly responsible for this down regulation.

Immunoblotting analysis determined that the molecular mass of the molecule recognised by 4H5 in HL-60 was approximately 160 kDa (Figure 3.5.). The g160 kDa polypeptide is probably a glycoprotein, as IFA with viable cells showed that the molecule is located on the surface of both parasite infected and HL-60 cells (Figure 2.1. and 3.2.). In agreement with the findings from *T. annulata* infected cells, recognition by monoclonal antibody 4H5 was sensitive to physical disruption by both denaturation and periodate treatment. Detection of TaHBL20/125 in *T. annulata* infected cell extracts was possible without periodate treatment, although periodation resulted in an quantitative increase in the level of polypeptide detected (see 2.3.2. and Figure 3.6.). In contrast, detection of the g160 kDa antigen on immunoblots of HL-60 was totally dependant on prior treatment with periodate (Figure 3.6.). As the HL-60 antigen was detectable by IFA under normal circumstances, it would seem to suggest that insertion into the cell membrane might bring about a conformational alteration to the g160 kDa molecule, allowing recognition by 4H5 without the requirement for periodation. Periodate oxidation of the native molecule on immunoblots would similarly appear to bring about structural modifications which facilitate antibody recognition. Immunoprecipitation experiments provided indirect evidence to support this theory, in that despite numerous attempts, the g160 kDa molecule could not be reproducibly immunoprecipitated from HL-60 cell extracts (data not shown). Detergents in the extracts could have altered the conformation of the antigen resulting in the failure of the antibody to recognise the g160 kDa antigen. Therefore, although recognition by monoclonal antibody 4H5 of antigens in both *Theileria*



infected and HL-60 cells is sensitive to treatments which affect structural integrity, identification of the g160 kDa antigen in HL-60 appears to be more susceptible to this effect.

IFA studies showed that differentiation of HL-60 cells was associated with increased reactivity with monoclonal antibody 4H5. This result was confirmed by immunoblotting, where progression towards granulocyte differentiation was associated with a quantitative increase in the band intensity of the g160 kDa polypeptide relative to the band representing  $\beta_2$ -microglobulin (Figure 3.7). Although this increase could reflect post translational changes to the molecule following differentiation, a more likely explanation is that levels of the g160 kDa antigen are up-regulated during differentiation.

In *T. annulata* infected cell lines the molecule recognised by the monoclonal antibody 4H5 was found to be 125 kDa. The difference in mass of the antigens recognised by 4H5 between TaHBL20 and HL-60 cells could indicate that the monoclonal antibody is cross reacting with a conserved epitope on unrelated polypeptides. However, the antigen detected by monoclonal antibody 4H5 is known to have a variable molecular mass on different *T. annulata* infected cell lines, probably as result of differential glycosylation (see 2.4.), and differential glycosylation has been well documented for surface molecules on cells of hematopoietic lineage, including HL-60 (Carlsson and Fukuda, 1986a; Carlsson, *et al.*, 1986b).

The extensive literature published on HL-60 was examined for an antigen which displays similarities to the g160 kDa polypeptide detected by monoclonal antibody 4H5. Antibodies directed against the CD66 cluster of differentiation antigens recognise a membrane bound polypeptide on the

surface of human granulocytes with a relative molecular mass of 160 kDa (Stoffel, *et al.*, 1993). This antigen is a product of the biliary glycoprotein-1 gene (BGP-1) and belongs to the CEA gene family. CD66 is expressed on mature neutrophils, some bone marrow progenitor cells and the brush border of colonic epithelium (Watt, *et al.*, 1991). When transfected into Chinese hamster ovary cells, CD66 has been shown to mediate homotypic adhesion (Watt, *et al.*, 1993). In the preceding chapter members of the CEA gene family were discussed as possible candidates for the identity of TaHBL20/125. Members of the CEA are difficult to characterise as monoclonal antibodies tend to cross react with other members of the family. Therefore, monoclonal antibody 4H5 may recognise conserved epitopes on related molecules in *T. annulata* infected cells and HL-60 cells.

The identity of the antigen recognised by monoclonal antibody 4H5 in both *Theileria* infected and HL-60 cells is unknown. It is also unknown whether the two antigens are directly related or whether they represent different molecules with shared epitopes for the antibody. Confirming the identity of each antigen by cloning the respective genes could provide information on the possible role of the molecule following infection by the parasite or during differentiation towards granulocytes. In addition, it would also allow studies to investigate and compare the mechanisms which control up-regulation of host cell gene expression by the parasite and up-regulation of HL-60 antigens during differentiation towards granulocytes.

## **Chapter 4**

## Studies on differentiation in HL-60

### 4.1. Introduction

The preceding chapter described how monoclonal antibody 4H5 recognised a 160 kDa antigen in the HL-60 cell line. The g160 kDa antigen was found to be lineage specific, as significant increase in expression occurred following the induction of differentiation towards granulocytes but not macrophages. Recognition of the g160 kDa antigen by monoclonal antibody 4H5 could therefore be used to extend previous investigations into the parallels between differentiation in *Theileria* (see 1.8.) and higher eukaryotic cells.

Despite the use of HL-60 cells as a widely studied model of human myeloid differentiation, the molecular mechanisms underlying these processes remain largely unknown. Studies on the differentiation of myeloid cells have shown that a large number of immediate early response genes, activated in the absence of *de novo* protein synthesis, occur following the induction of differentiation (Lord, *et al.*, 1990). Examples include transient induction of ICAM-1 (Lord, *et al.*, 1990), alterations in the expression of various proto-oncogenes (Antoun, *et al.*, 1991; Boise, *et al.*, 1992a; Collins, 1987; Holt, *et al.*, 1988; Lord, *et al.*, 1990; Wu, *et al.*, 1990) and transcription factors (Mollinedo, *et al.*, 1993; Nguyen, *et al.*, 1993; Sokoloski, *et al.*, 1993) and the increased expression of histone genes (Lord, *et al.*, 1990). Some of these immediate early genes have been found to be restricted to a particular lineage, while the expression of others seems to be inducer specific. In most cases, the precise role that these early alterations play in the process of differentiation is unclear.

The transduction of specific kinase signalling pathways have been implicated in the mechanism of macrophage differentiation, as TPA is known to be an activator of protein kinase C which acts immediately upstream of the cytoplasmic Raf-1 serine/threonine protein kinase (Katagiri, *et al.*, 1994; Kharbanda, *et al.*, 1994; Matikainen and Hurme, 1994; Pilz, *et al.*, 1994). A number of macrophage specific transcription factors have been isolated which could be important in the determination of the HL-60/macrophage differentiation step. For example, anti-sense oligonucleotides directed against the Egr-1 transcription factor have the ability to block macrophage differentiation in response to PMA, while constitutive expression of an *egr-1* transgene in undifferentiated HL-60 cells blocks DMSO induced granulocytic differentiation, but not the formation of macrophages (Nguyen, *et al.*, 1993). Mollinedo, *et al.* (1993) suggested that qualitative and quantitative differences in the expression of another transcription factor, AP-1, may be important in the regulation and restriction of monocyte/macrophage and granulocytic differentiation. The commitment stage of macrophage differentiation was found to be associated with an increase in the mRNA levels of *jun B* and *c-fos*, and correlated to an increase in AP-1 activity (Mollinedo, *et al.*, 1993). Differentiation along the granulocyte pathway however, did not result in changes in *c-fos* or *c-jun* expression, but was associated with an increase in the level of Jun B and Jun D proteins. The activity of AP-1 either decreased or remained unchanged during granulocytic differentiation. In the absence of c-Fos, Jun B and Jun D bind to DNA very poorly. A quantitative/qualitative model of controlling lineage specificity was suggested, where increases in the levels of these two proteins could serve to 'squench' the formation of c-Fos/c-Jun heterodimers during granulocytic differentiation.

Much work on HL-60 has been directed towards the role of various oncogenes during growth arrest and differentiation. For example, the expression of the *c-fms* oncogene has been found to be specifically associated with the formation of macrophages, as inhibition of this gene by anti-sense oligonucleotides significantly inhibits induction of macrophage differentiation (Wu, *et al.*, 1990).

Down regulation of some oncogenes has also been found in response to differentiation. DMSO induced differentiation of HL-60 is characterised by a rapid reduction in the levels of *c-myc* mRNA (Shima, *et al.*, 1989; Siebenlist, *et al.*, 1988; Van Roozendaal, *et al.*, 1990). Experiments using anti-sense RNA or oligonucleotides have shown that inhibition of *c-myc* translation results in a decrease in the growth rate of HL-60 cells and the expression differentiation markers (Bacon and Wickstrom, 1991; Holt, *et al.*, 1988; Yokoyama and Imamoto, 1987). However, when HL-60 cells are grown in serum-starved conditions and become non-proliferating, no reduction of *c-myc* steady state RNA levels are detected (Filmus and Buick, 1985). This suggests that other mechanisms are more directly involved in the control of proliferation in HL-60 *per se*, and that the observed reduction in *c-myc* expression subsequent to differentiation induction could be directly related to the control of proliferation during this differentiation process, rather than to a cell cycle-related phenomenon. There is also evidence that the rapid down regulation of *c-myc* in response to DMSO is not necessarily an obligatory step associated with granulocytic differentiation. When actinomycin D and novobiocin are used, no rapid decrease in *c-myc* is observed (Stocker, *et al.*, 1995). Similarly, when cells are induced towards granulocytes by retinoic acid, a more gradual decrease in *c-myc* takes place (Van Roozendaal, *et al.*, 1990).

A similar rapid down regulation of the *c-myb* oncogene is observed following the induction of differentiation, however re-expression subsequently takes place 6-24 hours later. Like *c-myc* the mechanism by which down regulation of *c-myb* is brought about has been shown to be agent specific (Boise, *et al.*, 1992b). Thus, as suggested by Van Roozendaal, *et al.* (1990), there is evidence that some of the immediate early responses to differentiation may be agent specific, indicating that multiple pathways may be operating prior to the attainment of terminal differentiation.

The immediate early associated gene, *mda-6* is found to be induced during differentiation of HL-60 cells along both the granulocyte and monocytic/macrophage pathway (Jiang, *et al.*, 1994). Mda-6 encodes a 21 kDa nuclear protein (p21) inhibitor of cyclin dependant kinase activity which has been proposed as having a more global role in the control of growth and differentiation. Moreover, a TPA resistant HL-60 cell line displayed a diminished response to the induction of *mda-6*, and structurally diverse inducers of differentiation were all able to bring about the expression of p21. However, more recent experiments demonstrated induction of p21 following monocyte/macrophage differentiation but did not demonstrate a similar induction of p21 following granulocytic differentiation (Schwaller, *et al.*, 1995) .

The findings described above would seem to suggest that alterations in the expression of individual genes induced early on in differentiation may not be central to the differentiation process, as changes in their expression do not always correlate with differentiation or with a specific cell lineage. It is possible, therefore, that some of these immediate early changes in gene expression could be a result of more global changes to the regulation of gene

expression which lead to specific alterations associated with the differentiation event.

The molecular mechanism by which DMSO exerts its effect and induces differentiation to granulocytes is unknown. A number of potential sites of action have been proposed, including suggestions that the cell membrane could be involved (Friend, *et al.*, 1971; Lyman, *et al.*, 1976). For example, exposure to DMSO was found to decrease the affinity of both insulin and granulocyte/macrophage colony stimulating factor for their respective receptors (Schwartz, *et al.*, 1993). There is also evidence that DMSO could have a general effect on membrane components, as incubation with DMSO has been shown to increase protein kinase C (PKC) activity in HL-60 cells (Durkin, *et al.*, 1992). However, as membrane activity of PKC only reaches a peak 10-20 minutes after the addition of DMSO, and DMSO does not stimulate inactive PKC when added to membranes from uninduced cells, it would seem unlikely that the increase in activity is a result of direct interaction and activation of membrane PKC (Durkin, *et al.*, 1992). In addition, the high concentrations and prolonged exposure times required to facilitate differentiation suggests that DMSO acts by a general mechanism, rather than by the activation of a specific signal transduction pathway (Breitman, *et al.*, 1980; Schwartz, *et al.*, 1993; Tarella, *et al.*, 1982). This possibility is supported further by the observation that DMSO is capable of inducing terminal differentiation in a number of other leukemic and transformed cell lines, implying interaction with a broad range of cellular molecules with low specificity (Friend, *et al.*, 1971; Huberman, *et al.*, 1979; Kimhi, *et al.*, 1976; Miranda, *et al.*, 1978).

Like some protozoan parasites and other eukaryotic cells (see 1.8.), studies have shown that differentiation of HL-60 to granulocytes is asynchronous



and stochastic, *i.e.* that differentiation occurs randomly with a predicted probability linked to culture conditions, is associated with a reduction in cell division (Boyd and Metcalf, 1984; Laskin, *et al.*, 1991; Tarella, *et al.*, 1982) and involves initial reversible events which lead to a commitment point (Tarella, *et al.*, 1982). A stoichiometric model based on similar observations in *T. annulata* has been proposed for differentiation to merozoites (see 1.8.). In this model it was postulated that commitment to differentiation was brought about by the build up of factors which regulate gene expression to a particular concentration threshold (Shiels, *et al.*, 1994). It was also proposed, from a comparison with the general cellular events of differentiation in higher eukaryotic cells, that the basic molecular mechanisms controlling differentiation were similar. Therefore, it was of interest to directly investigate parallels between differentiation in the parasite and HL-60 cells, using reactivity of monoclonal antibody in HL-60 as a sensitive marker of granulocytic differentiation.

#### 4.1.1. Aims

The specific aim of the work presented in this chapter was firstly, to use monoclonal antibody 4H5 reactivity against the g160 kDa antigen as a marker to investigate the induction of this molecule during the course of DMSO induced granulocytic differentiation in HL-60 cells. Secondly, in comparison with differentiation to the merozoite in *T. annulata*, to determine if a reversible phase was associated with the expression of this antigen early on in differentiation. Thirdly, based on knowledge that differentiation is often associated with a reduction in proliferative potential, and on the results of current research on the modulation of *T. annulata* differentiation by inhibition of DNA or protein synthesis, to test the possibility that altered growth conditions and cell proliferation/DNA synthesis could affect expression of the g160 kDa antigen.

## **4.2 Materials and Methods**

### **4.2.1. Cell lines and culture conditions**

HL-60 were maintained and induced to granulocytic differentiation as described previously (3.2.1.). Aphidicolin was dissolved as a stock in DMSO, 3  $\mu$ l of stock was added to cells resuspended at  $4 \times 10^5$  cells  $\text{ml}^{-1}$  in 10 ml of fresh medium to give a final concentration of  $1.5 \mu\text{g ml}^{-1}$ . Control cultures without aphidicolin contained a similar volume of DMSO.

### **4.2.3. Measurement of DNA synthesis**

The rate of DNA synthesis was measured by incorporation of [ $^3\text{H}$ ] thymidine into cells following culture in the presence or absence of DMSO or aphidicolin. Cells were resuspended at  $4 \times 10^5$  cells  $\text{ml}^{-1}$  in normal medium containing 6  $\mu\text{Ci ml}^{-1}$  of [ $^3\text{H}$ ] thymidine. Six wells of a Linbro multiwell, plate containing 1 ml of resuspended cells, were set up for each time point and the plates incubated at  $37^\circ\text{C}$  for 5 hours. Following labelling, cells were washed three times with PBS and the reaction terminated by the addition of 500  $\mu$ l of 11% TCA. The precipitate was collected onto microglass fibre filters, washed with 5% TCA, 100% ethanol and acetone, and counted on a Packard 1600 TR liquid scintillation analyser. DNA synthesis was expressed as the mean counts per minute (cpm) per  $4 \times 10^5$  cells of the six wells for each time point.

### **4.2.2. Measurement of protein synthesis**

The rate of protein synthesis was measured by incorporation of [ $^{35}\text{S}$ ] methionine into TCA insoluble material. 10 ml cultures were established in the presence or absence of DMSO or aphidicolin, and cultured as described previously (see 3.2.1.). At appropriate time points samples were taken and the cells resuspended at  $4 \times 10^5$  cells  $\text{ml}^{-1}$  in methionine free medium in the

presence of 14  $\mu\text{Ci ml}^{-1}$  [ $^{35}\text{S}$ ] methionine and 1.3% DMSO. Six wells of a multiwell plate, containing 1 ml of resuspended cells, were set up for each time point and the plates incubated at 37°C for 5 hours. Cells were washed three times with PBS and lysed by the addition of 100  $\mu\text{l}$  of NET buffer (100 mM Tris. HCl, pH 8.0, 0.5% NP-40, 1 mM EDTA), followed by repeated freeze thawing on dry ice. Extracts were centrifuged at 15 800 g for 15 minutes to remove cell debris and a 10  $\mu\text{l}$  aliquot precipitated using 100  $\mu\text{l}$  of 11% TCA for 30 minutes. The precipitate was then collected onto microglass fibre filters, washed with 11% TCA, 100% ethanol and acetone and scintillation counted as described above. Protein synthesis was expressed as the mean cpm per  $4 \times 10^5$  cells of the six wells for each time point.

## 4.3 Results

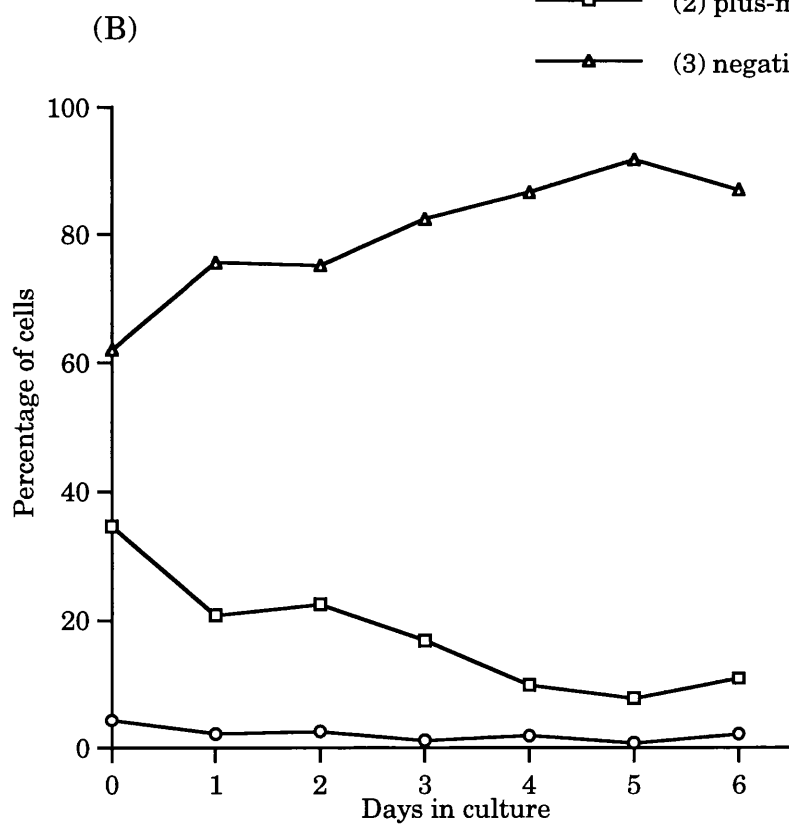
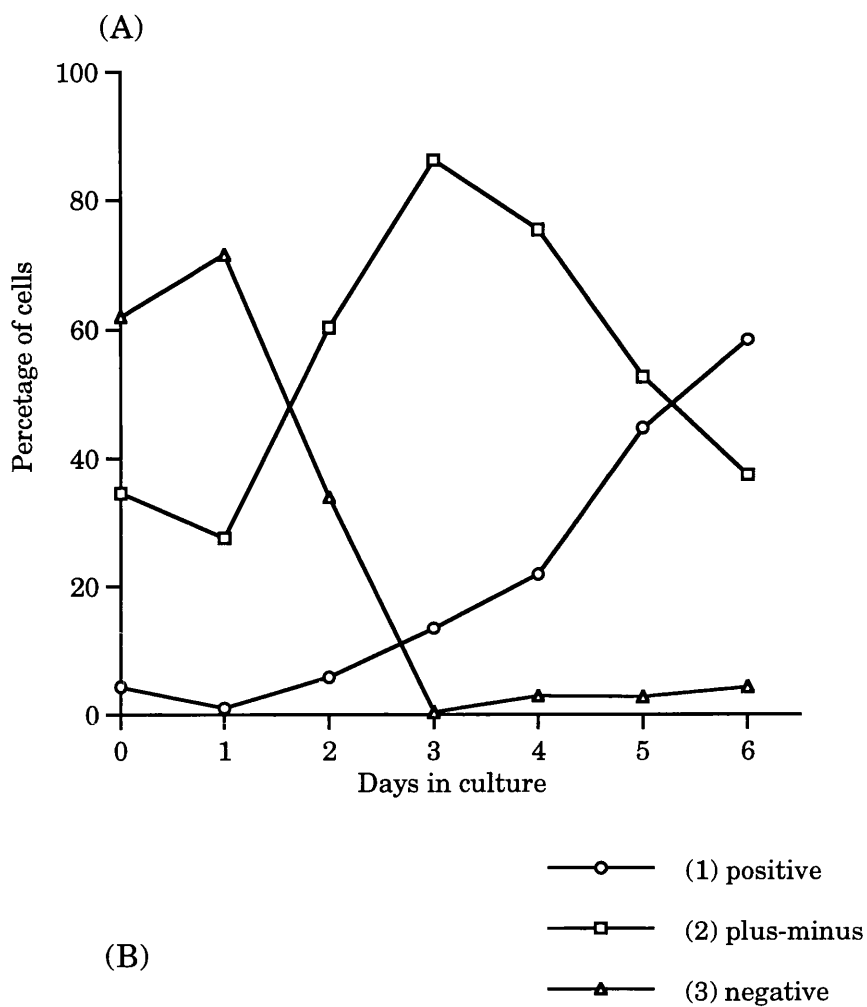
### 4.3.1. Kinetics of 4H5 reactivity during differentiation towards granulocytes

In HL-60, expression of the g160 kDa molecule recognised by monoclonal antibody 4H5 was elevated following differentiation towards granulocytes. By using 4H5 reactivity as a marker, a closer examination of the process of differentiation was undertaken. Expression of the 4H5 epitope was analysed quantitatively by grading reactivity with the antibody as positive, showing intense fluorescence; plus-minus, showing intermediate reactivity; or negative, showing no reactivity (as indicated in Figure 4.4.B).

The majority of HL-60 cells (62 to 92%) showed no detectable reactivity under normal culture conditions (Figure 4.1.B). Following the addition of DMSO, the level of reactivity was clearly altered (Figure 4.1.A). After 24 hours there was a cumulative increase in the number of plus-minus cells. This increase continued until day 3, when 86% of the cells were of this type. A reduction in the percentage of negative cells was concomitant with the increase in plus-minus cells, with a cross over occurring between the two cell types at day 2. Between day 3 and day 6, there was a reduction in the number of cells with plus-minus reactivity and a significant increase in the positive cell population to a peak of 58% a day 6. These two cell populations crossed over at day 5. The negative cell population continued to decline until day 3, where there was a small increase in numbers, probably as a result of the proliferation of undifferentiated cells in the culture.

In control cultures containing no DMSO, the populations of negative and plus-minus cells showed small variations throughout the time course (Figure 4.1.B). Generally, negative cells were found to slightly increase

**Figure 4.1.** Pattern of reactivity of HL-60 cells with monoclonal antibody 4H5 by immunofluorescence during; (A) 6 days of continuous exposure to 1.3% DMSO and (B) a control culture without DMSO. Three cell types were recorded; (1) intensely fluorescent positive cells; (2) weakly reactive plus-minus cells and (3) non reactive negative cells.



and plus-minus cells were found to slightly decrease. These two cell populations appeared to have an inverse relationship to each other, and this was also seen in subsequent experiments. The percentage of positive cells in the control culture remained at a low level throughout the experiment.

A similar experiment determined that the kinetics of 4H5 reactivity on HL-60 cells incubated in DMSO correlated with granulocyte formation. Cells were assessed at daily intervals until day 6 for both 4H5 reactivity and NBT reduction. An increasing proportion of terminally differentiated cells was observed up to day 5, where the percentage of 4H5 positive cells reached a peak of 68% (Figure 4.5.C) and the percentage of NBT positive cells reached a peak of 83%. Thus, the increase in NBT positive cells up to day 5 (Figure 4.6.) was, in general, parallel to the increase in the number of cells showing positive reactivity with 4H5. This result verified that positive reactivity with 4H5 correlated with differentiation to granulocytes. In addition, these results also indicated that a small number of undifferentiated cells were present in the culture even after five days of continuous exposure to DMSO.

#### **4.3.2. Reversible expression of the g160 kDa antigen on HL-60**

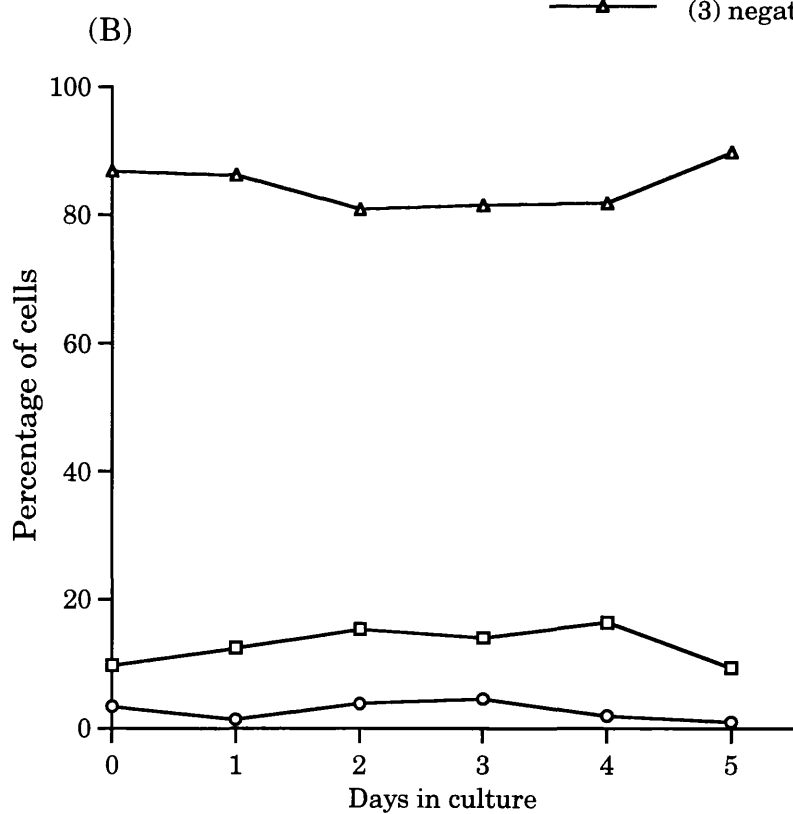
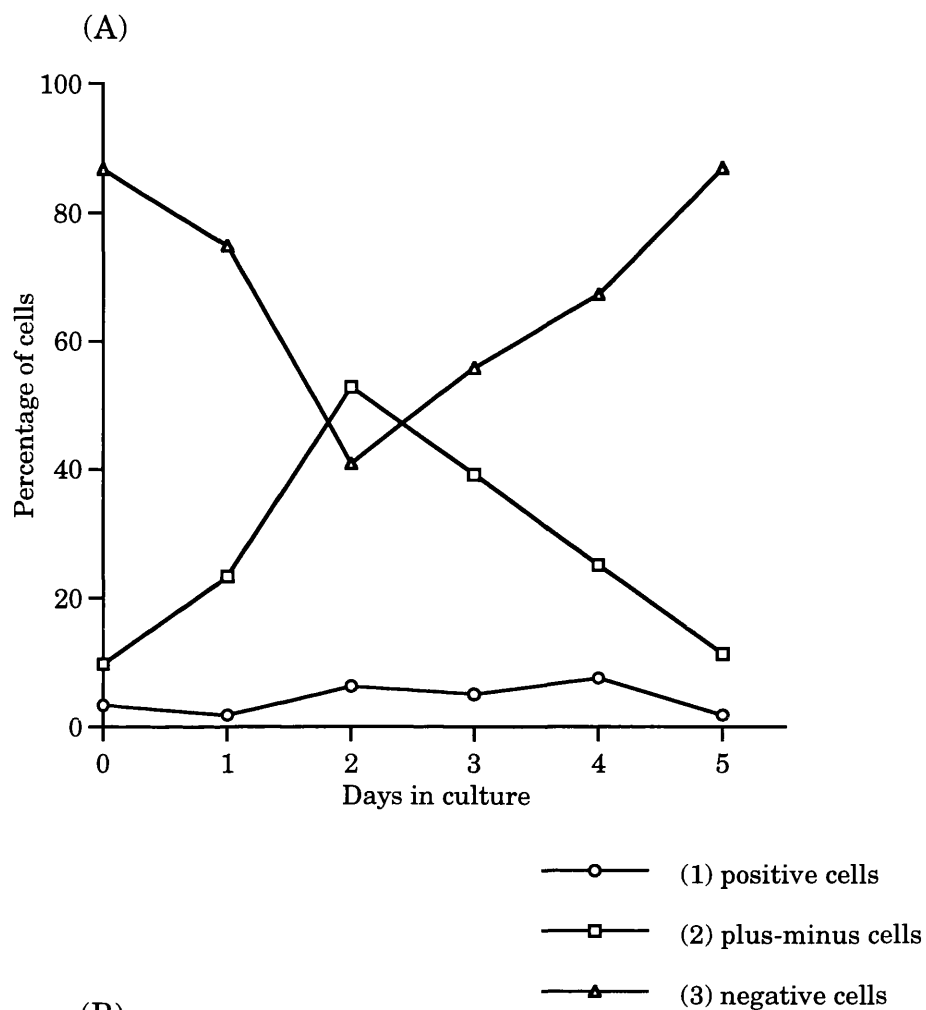
Differentiation has been shown to involve an intermediate reversible phase prior to commitment in both parasites, including *Theileria*, and in higher eukaryotes, including HL-60 (see 1.8.). From the cross over kinetics observed between the three cell populations, it was possible to conclude that DMSO induced granulocyte differentiation occurred via an intermediate stage, and that progression through this stage was associated with a quantitative increase in level of the antigen detected by monoclonal 4H5.



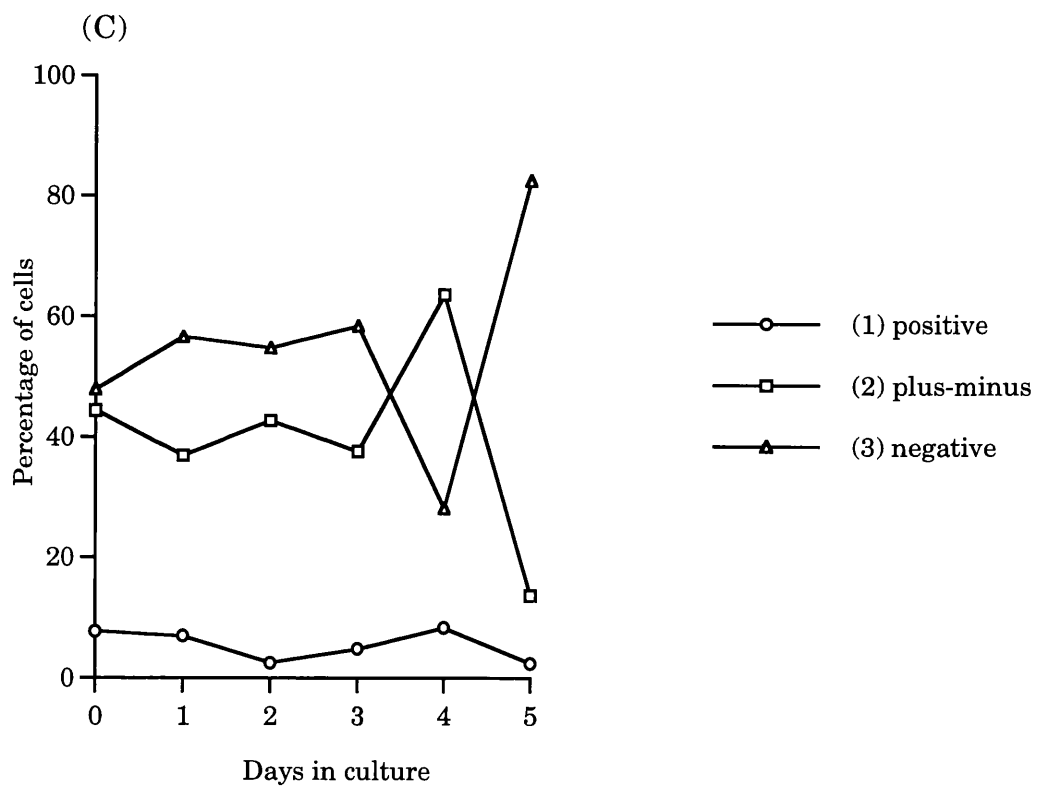
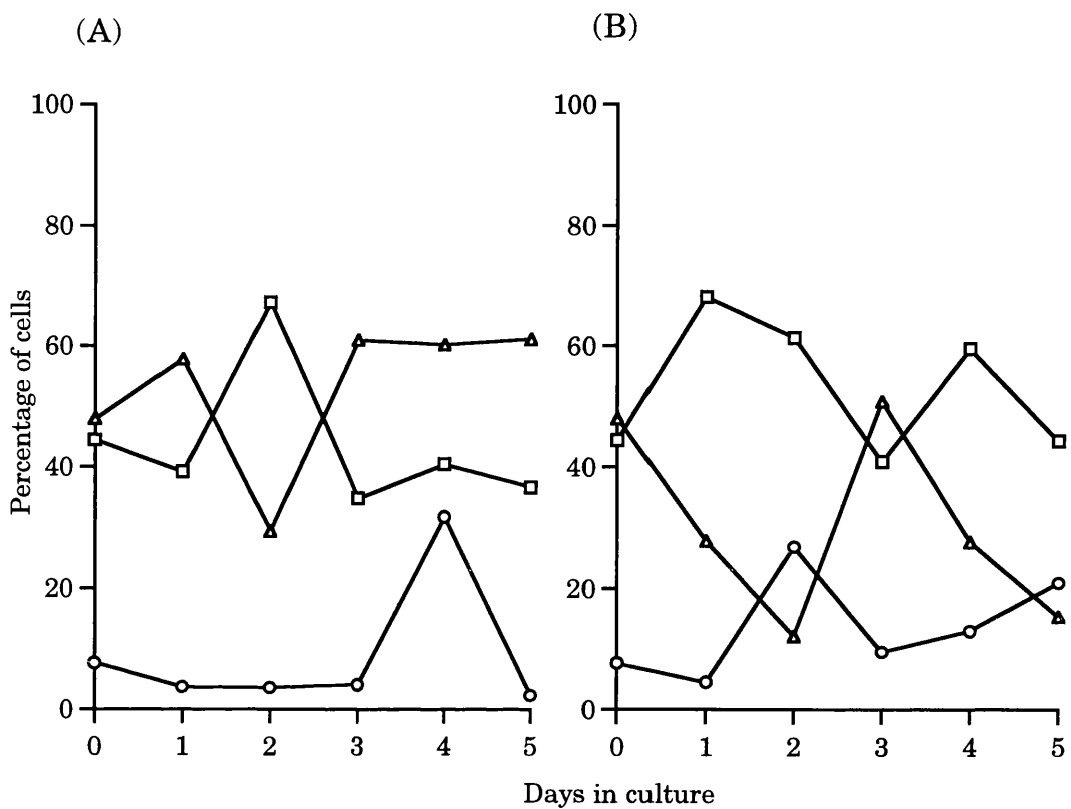
To investigate whether expression of the 4H5 antigen correlated with commitment to differentiation, cells were incubated with 1.3% DMSO for 18 hours, washed and resuspended in DMSO free medium for the remainder of the incubation period. The cultures were assessed for monoclonal antibody 4H5 reactivity every day for 5 days and cells scored as positive, plus-minus or negative (Figure 4.2.A). Incubation of cells with DMSO for 18 hours resulted in an initial increase in the percentage of plus-minus cells to a peak of 53% at day 2. The increase was then followed by a steady decline of this population until day 5, where the number of plus-minus cells was approximately the same as estimated at day 0. The pattern observed for the plus-minus population was found to be inversely related to the negative population, as the number of negative cells initially declined to a trough of 41% at day 2, but was followed by a steady recovery until the numbers at day 5 were approximately equal to those estimated at day 0. This inverse relationship was manifest as biphasic kinetics where just prior to day 2, a cross over occurred between the plus-minus and negative cell populations which crossed back approximately 24 hours later. In contrast to the biphasic kinetics observed for the negative and plus-minus populations, the numbers of positive cells remained more or less constant throughout the 5 day period, although generally the numbers were slightly elevated when compared to the control (Figure 4.2.B).

The proportions of the three cell types during shorter and longer exposures to DMSO was also investigated by a second set of DMSO pulse experiments. HL-60 cells were induced for varying lengths of time from 6 to 96 hours, after which time cells were washed and resuspended in DMSO free medium for the remainder of the experiment. The cultures were assessed for monoclonal antibody 4H5 reactivity every day for 5 days and cells scored as positive, plus-minus or negative (Figures 4.3.1. and 4.3.2.). Incubation times

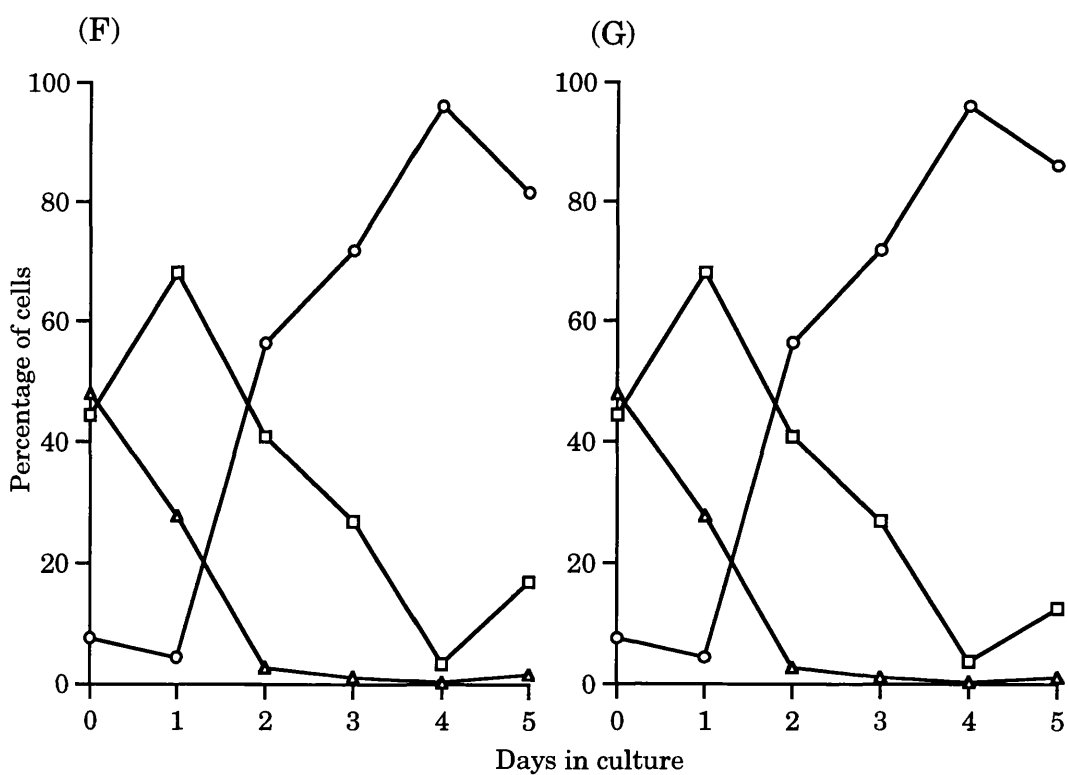
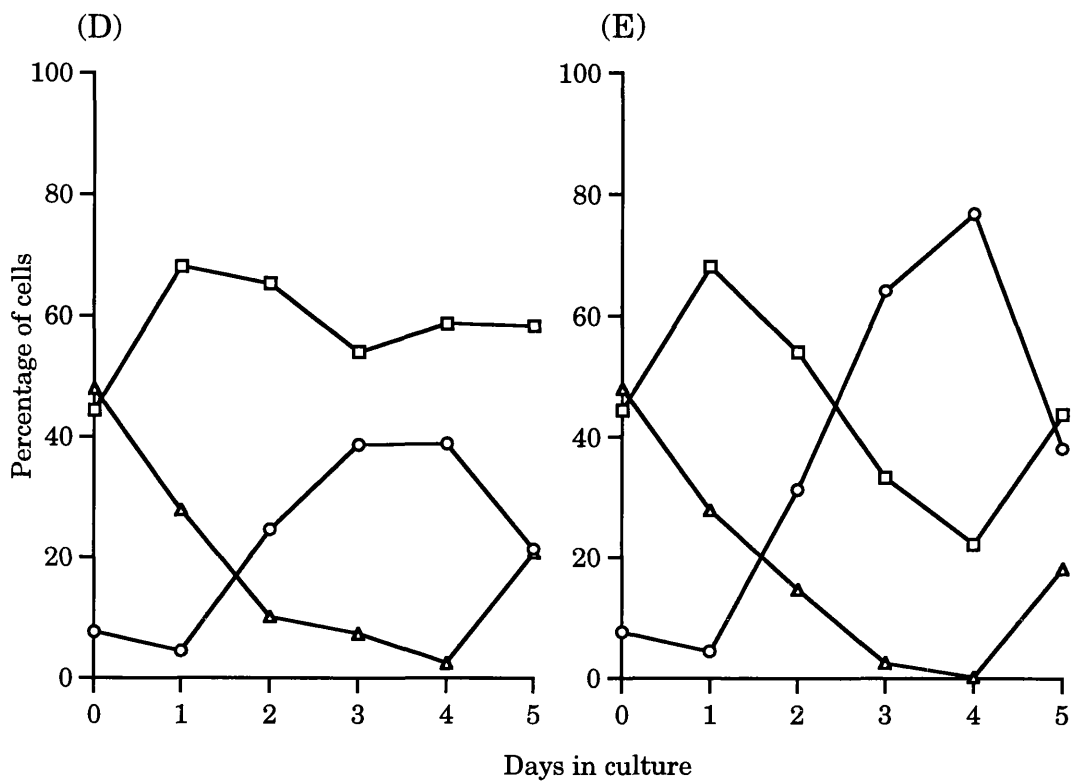
**Figure 4.2.** Pattern of reactivity of HL-60 cells with monoclonal antibody 4H5 by immunofluorescence over a 5 day period following; (A) 18 hour incubation with 1.3% DMSO. After 18 hours the cells were washed and incubated in normal medium for the remainder of the experiment; (B) control cultures containing no DMSO. Three cell types were recorded; (1) intensely fluorescent positive cells; (2) weakly reactive plus-minus cells and (3) non reactive negative cells.



**Figure 4.3.1.** Immunofluorescence reactivity of monoclonal antibody 4H5 on fixed slide preparations of HL-60 cell incubated with 1.3% DMSO for; (A) 6 hours; (B) 18 hours and (C) control cultures containing no DMSO. After incubation in DMSO the cells were washed and incubated in normal medium for the remainder of the experiment. Three cell types were recorded; (1) intensely fluorescent positive cells; (2) weakly reactive plus-minus cells and (3) non reactive negative cells.



**Figure 4.3.2.** Immunofluorescence reactivity of monoclonal antibody 4H5 on fixed slide preparations of HL-60 cells incubated with 1.3% DMSO for; (D) 24 hours; (E) 36 hours; (F) 72 and (G) 96 hours. After incubation in DMSO the cells were washed and incubated in normal medium for the remainder of the experiment. Three cell types were recorded; (1) intensely fluorescent positive cells; (2) weakly reactive plus-minus cells and (3) non reactive negative cells.



- (1) positive
- (2) plus-minus
- ▲— (3) negative

below 24 hours produced unexpected variations in the three cells types. Furthermore, the control culture displayed large fluctuations in the populations of negative and plus minus cells after day 3 (Figure 4.3.1.C), whereas in previous experiments only relatively small variations had been observed (Figures 4.1.B and 4.2.B). A 6 hour incubation with DMSO was not expected to have much effect on the differentiation of HL-60, however, in addition to the large fluctuations observed in the negative and plus-minus populations, there was an increase in the numbers of positive cells at day 4 (Figure 4.3.1.A). Similarly, the pattern of expression observed at 18 hours incubation (Figure 4.3.1.B) was not consistent with previous results (4.2.A).

Incubation times of 24 hours or longer tended to follow the pattern of expression predicted from previous experiments. Induction of differentiation with DMSO for 24 hours resulted in an initial increase in the percentage of plus-minus cells to a peak of 68% at day 1, followed by a more gradual decline thereafter (Figure 4.3.2.D). This decline was associated with an increase in the percentage of positive cells to a peak of 39% at day 3 to 4 and a steady decrease in the percentage of negative cells to day 4. Significantly, there was no cross back due to increasing negative and declining plus-minus populations, similar to that observed with the 18 hour DMSO incubation represented by Figure 4.2.A. Between day 1 and day 2, the declining negative and elevating positive cell populations cross over, however the cross over of decreasing plus-minus and increasing positive cell populations as observed in cultures exposed to DMSO for longer periods (Figure 4.3.2. E, F and G) of time did not occur. At day 5 there was an increase in the numbers of negative cells and a decrease in the numbers of positive cells. This was probably a result of proliferation of undifferentiated cells in the culture as previously described by Tarella, *et al.* (1982).



Induction of differentiation for longer periods in DMSO, 36 to 96 hours (Figure 4.3.2.E, F and G), resulted in similar patterns of expression for the three cell types and was consistent with patterns obtained following continuous exposure (Figures 4.5.C and 4.1.A). Incubation of cells with DMSO for 36 hours resulted in a smaller percentage of positive cells (77%) compared to cells incubated for 72 and 96 hours (96%). This indicated that, generally, the longer incubation times resulted in greater numbers of positive cells, but only up to a threshold of 72 hours. After this point further incubation in the inducer had no effect on the peak number of positive cells as commitment to differentiation had presumably reached the maximum. At day 5 following incubation in DMSO for 36 hours an increase in the percentage of negative and plus cell types and a decline in the percentage of positive cells was seen (Figure 4.3.2.E.). This was probably the result of the proliferation of undifferentiated cells in the culture as discussed previously. However, the recovery in numbers of negative and plus-minus cell types and decline of positive cells was markedly greater than those observed at the same time point following incubation in DMSO for 72 and 96 hours, and could reflect the greater proportion of cells committed to terminal differentiation in the cultures at the longer incubation times.

Pulse experiments described above lead to the hypothesis that culture conditions could be having an effect on expression of the g160 kDa antigen with DMSO exposure times below 24 hours, thus altering the proportions of the three designated cell types. To determine directly whether changes in growth altering conditions could modulate monoclonal 4H5 reactivity, cultures were either left for 5 days without dilution or maintained at the same cell number throughout this time period. Figure 4.4.A. compared to

**Figure 4.4.** Immunofluorescence reactivity of monoclonal antibody 4H5 on fixed slide preparations of; (A) HL-60 cells maintained in cell culture by daily replacement of medium and (B) HL-60 cells maintained for a period of 5 days without any replacement of medium. (Bar = 10  $\mu\text{m}$ )

Examples of cells classified as showing; intense positive (P), intermediate/plus-minus (I) and negative (N) fluorescence reactivity are indicated in panel B.

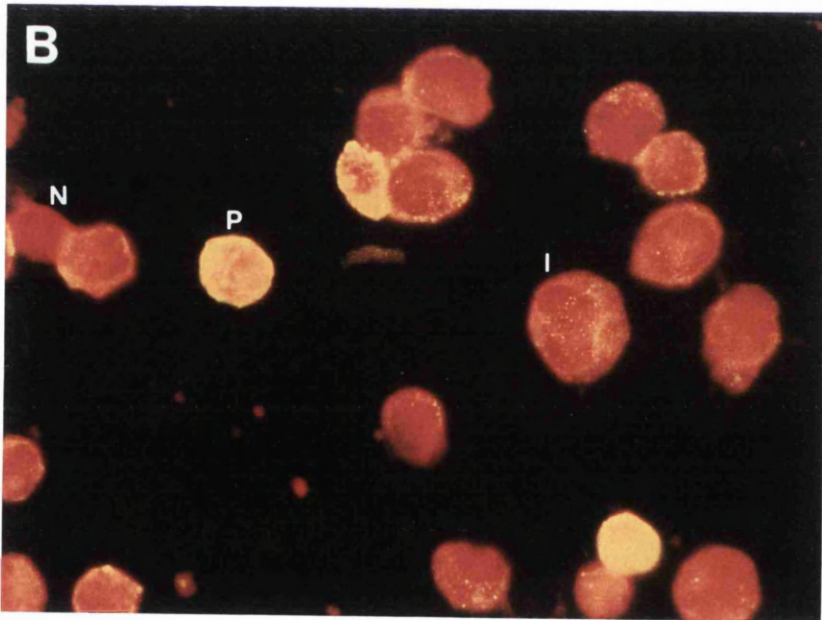
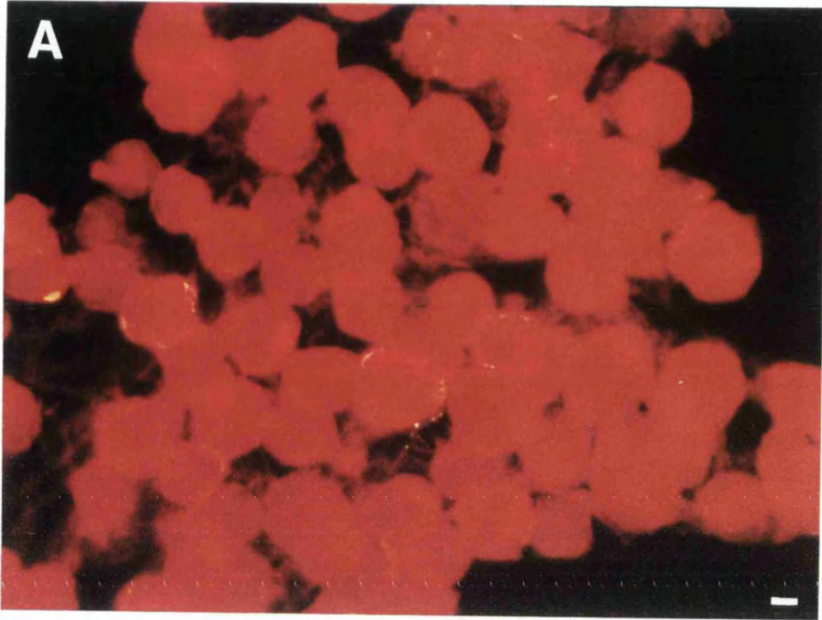
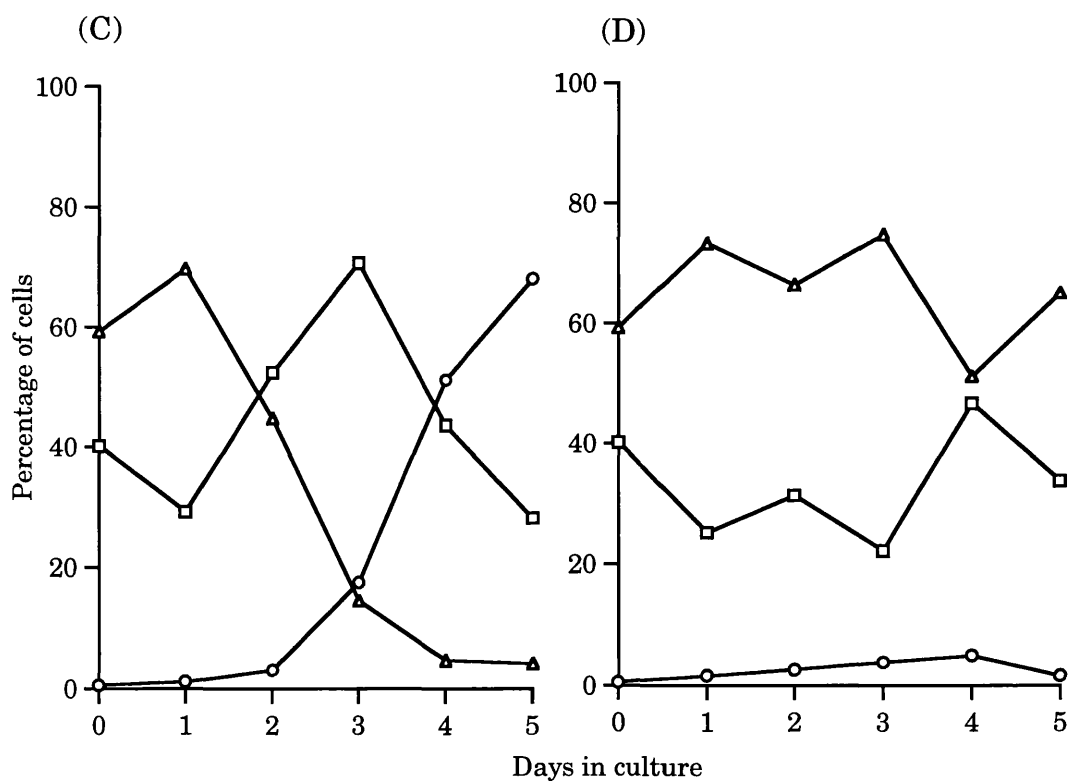
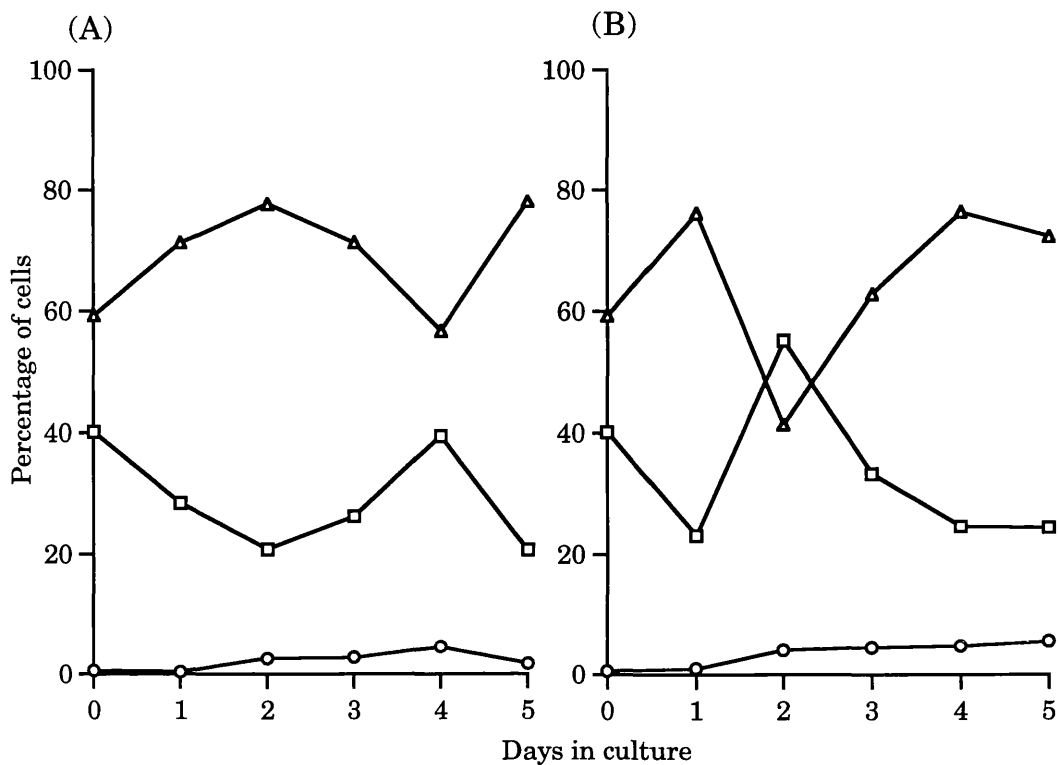


Figure 4.4.B showed that, cultures which remained undiluted for 5 days contained a greater number of 4H5 positive cells than cultures where the cell number was maintained. In view of this result it was thought that at the low DMSO pulse times, expression of g160 kDa antigen could have been influenced by alterations due to fluctuations in cell number. Therefore, the short DMSO pulse experiments were repeated using cultures where the cell numbers were maintained throughout the experiment (Figure 4.5.). In both the negative control (Figure 4.5.D) and 6 hour (Figure 4.5.A) cultures a variation in the number of negative and plus-minus cells was observed during the time course, however there was no cross over between increasing and decreasing populations. In addition, a large rise in positive cells was not observed. Incubation with DMSO for 12 hours (Figure 4.5.B.) resulted in similar kinetics to those described for the 18 hour incubation (Figure 4.2.A). There was no great rise in the number of positive cells, but the biphasic relationship between the plus-minus and the negative cell populations was observed (Figure 4.5.B).

#### **4.3.3. Elevation of g160 kDa expression by modulation of growth relative to division**

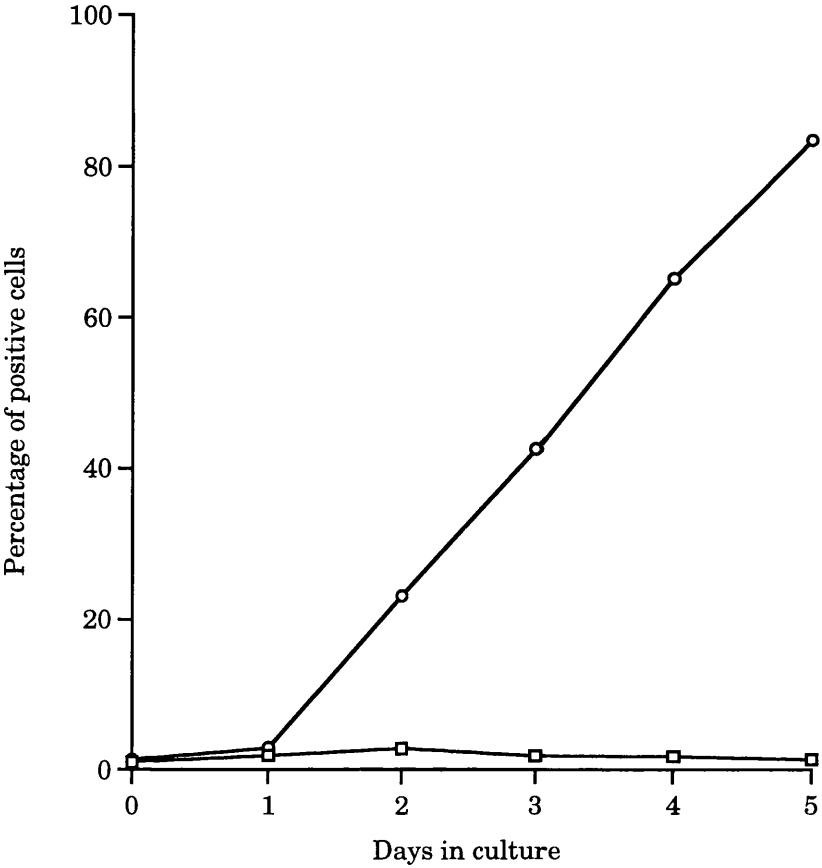
The experiment described above demonstrated that in the absence of an inducer, differentiation to granulocytes could be brought about by alterations in growth conditions. Similar results have been obtained with differentiation of *T. annulata* infected cells, as alteration of the cell number has been shown to modulate the level of merozoite formation at 41°C (Shiels and McKellar, unpublished data). The effect of reduced proliferation on *Theileria* differentiation has also been investigated directly by the addition of aphidicolin, an inhibitor of DNA polymerase alpha (see 4.4.). To compare the HL-60 and *Theileria* systems it was investigated whether reduced division influenced 4H5 antibody reactivity following incubation of HL-60

**Figure 4.5.** Immunofluorescence reactivity of monoclonal antibody 4H5 on fixed slide preparations of HL-60 cells incubated with 1.3% DMSO for; (A) 6 hours; (B) 12 hours; (C) continuous exposure and (D) control cultures containing no DMSO. Cell numbers were maintained at a density of  $4 \times 10^5$  cells  $\text{ml}^{-1}$  by daily sub-culturing. After incubation in DMSO the cells were washed and incubated in normal medium for the remainder of the experiment. Three cell types were recorded; (1) intensely fluorescent positive cells; (2) weakly reactive plus-minus cells and (3) non reactive negative cells.



- (1) positive  
—□— (2) plus-minus  
—▲— (3) negative

**Figure 4.6.** Differentiation pattern of HL-60 cells detected during 5 days of continuous exposure to DMSO. Cell numbers were maintained at a density of  $4 \times 10^5$  cells ml<sup>-1</sup> by daily sub-culturing. Differentiation is expressed as the percentage of NBT positive cells present in; (1) control cultures without DMSO or (2) cultures continuously exposed to 1.3% DMSO.



—□— (1) control culture

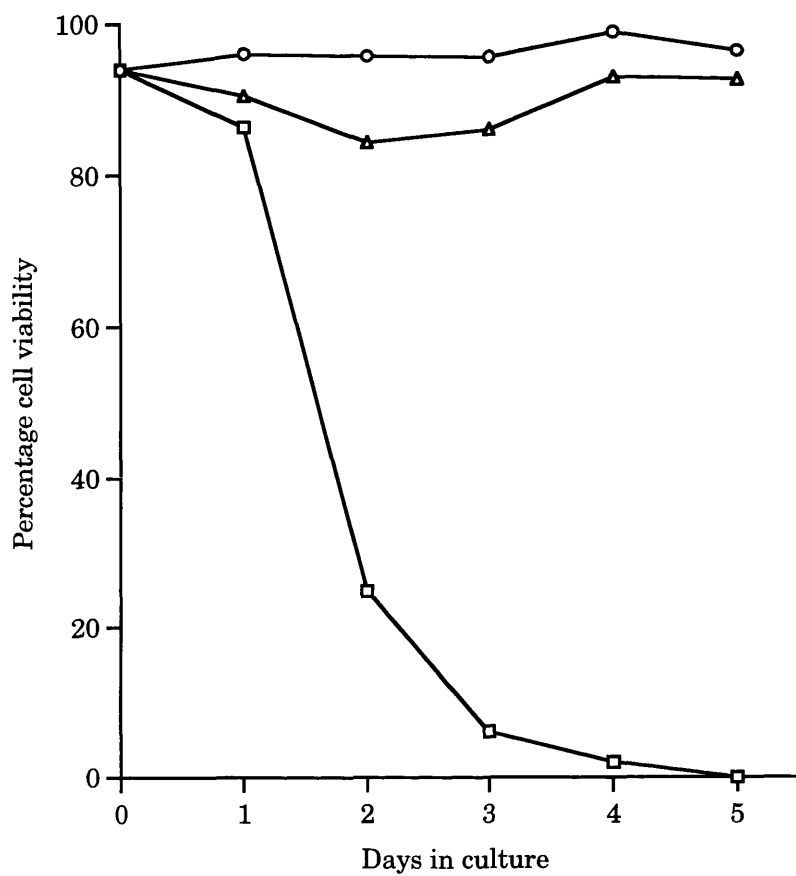
—○— (2) granulocyte culture



cultures with aphidicolin. Preliminary experiments showed that cultures maintained in the presence of the drug for periods in excess of 24 hours resulted in significant loss of cell viability (Figure 4.7.). To overcome this detrimental effect, cells were incubated for 18 hours, and subsequently cultured in drug free medium. The result of this experiment showed that incubation in the drug increased the percentage of positive cells to 5.8 %, which was 4 fold greater than the control at day 1 (Figure 4.8.A). This increase continued to a peak of 9.1 % at day 2 (2.4 fold increase over control culture). By 72 hours, however, the value for the aphidicolin treated culture was reduced and was approximately the same as estimated for the control. These results were essentially mirrored by counting the number of plus-minus cells following drug treatment (Figure 4.8.B), except that the plus-minus cells represented a greater proportion of the total population of the culture (24.7 % and 56.24 % at day 1 and 2), and a significant increase between the number of plus-minus cells in the drug treated culture relative to the control was maintained to day 3. From these results it was concluded that aphidicolin could induce cells to increase 4H5 antigen expression, but elevation to the level associated with commitment only occurred for a minority of the population.

To confirm the effect of aphidicolin on DNA synthesis in HL-60, cells were incubated for 13 hours in aphidicolin and incorporation of [<sup>3</sup>H] thymidine and [<sup>35</sup>S] methionine estimated over a 5 hour period in the presence of the drug. This experiment demonstrated that relative to the control cultures, aphidicolin inhibited the rate of DNA synthesis by 94% (Table 4.1.). In contrast the drug had a relatively minimal effect on protein synthesis when incubated with the cells for 18 hours (2.9% reduction). Thus, over the incubation periods used in this study aphidicolin was found to preferentially inhibit DNA synthesis relative to protein synthesis.

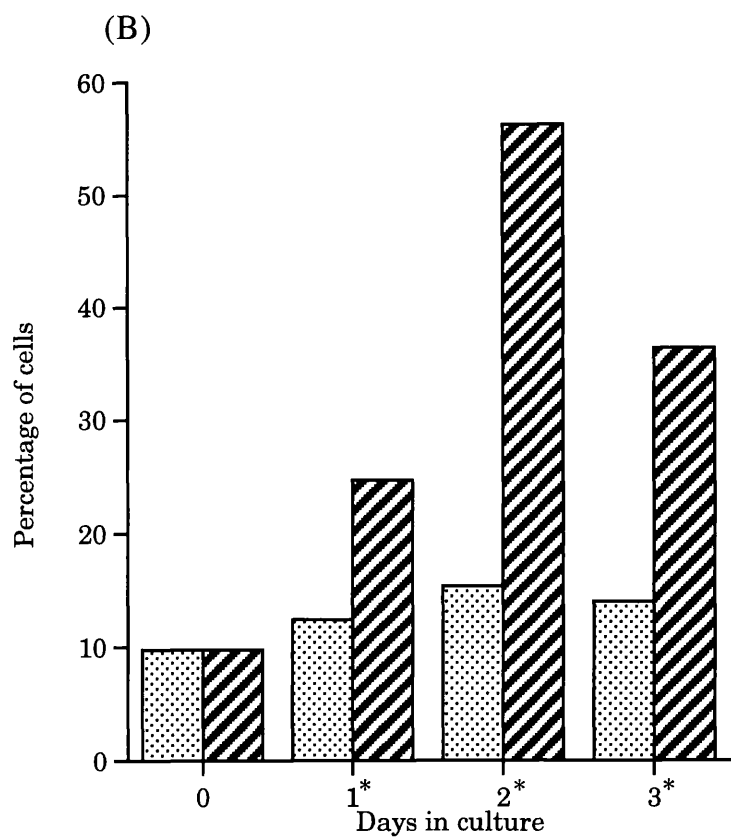
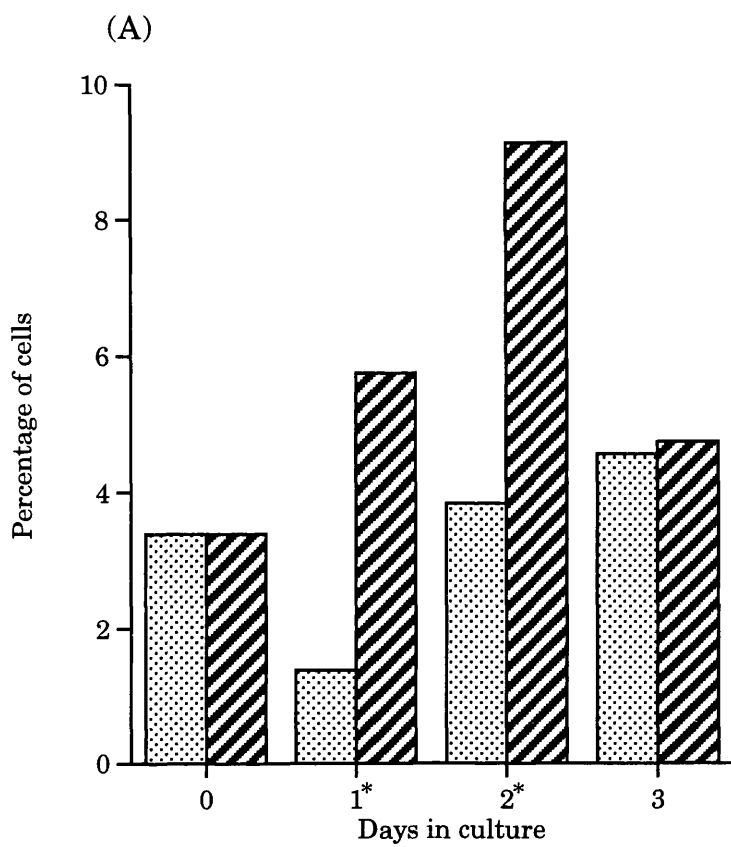
**Figure 4.7.** Effect of aphidicolin on the viability of HL-60 cells. Cultures were assessed for cell viability every day for 5 days using trypanblue exclusion (50% in PBS). Cultures were incubated with; (1) no drug, control cultures; (2)  $1.5 \mu\text{g ml}^{-1}$  aphidicolin for 18 hours after which time the cells were washed and incubated in normal medium for the remainder of the experiment; (3) continuous exposure to  $1.5 \mu\text{g ml}^{-1}$  aphidicolin.



- (1) control
- ▲— (2) 18 hours aphidicolin
- (3) aphidicolin

**Figure 4.8.** Effect of aphidicolin on differentiation of HL-60. Cells were incubated with; (1) no drug, stippled bar or (2)  $1.5 \mu\text{g ml}^{-1}$  aphidicolin, hatched bar. After 18 hours the cells were washed and incubated in normal medium for the remainder of the experiment. Graph (A) percentage of cells which gave positive reactivity by immunofluorescence with monoclonal antibody 4H5. Graph (B) percentage of cells which gave (+/-) reactivity with monoclonal antibody 4H5.

\* Denotes time points where a statistically significant difference ( $p < 0.01$ ) was observed between control and drug treated cultures. Statistical analysis was carried out using the  $\chi^2$  distribution and 2 x 2 contingency tables (Harper, 1971).



■ (1) control      ■ (2) aphidicolin

**Table 4.1.** Effect of aphidicolin on protein and DNA synthesis in HL-60 cells. Cultures were incubated for a total of 18 hours in the presence of 1.5 µg ml<sup>-1</sup> aphidicolin or control cultures containing no drug. Incorporation of either [<sup>35</sup>S] methionine or [<sup>3</sup>H] thymidine was estimated during the final 5 hours of incubation.

	[ <sup>35</sup> S] Incorporation	% Inhibition	[ <sup>3</sup> H] Incorporation	% Inhibition
Control	2204170 (± 327846)	2.9	17306 (± 2179)	94.2
Aphidicolin	2139288 (± 307658)		1001 (± 162)	

Values are the mean counts per minute per 10<sup>5</sup> cells of 6 sample wells, +/- the standard deviation.

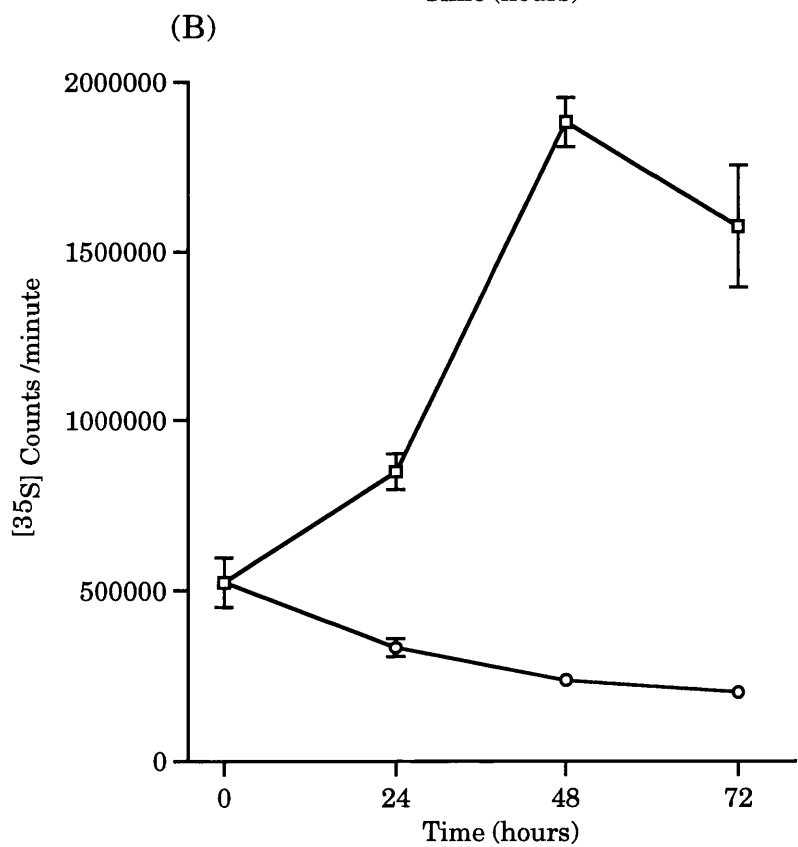
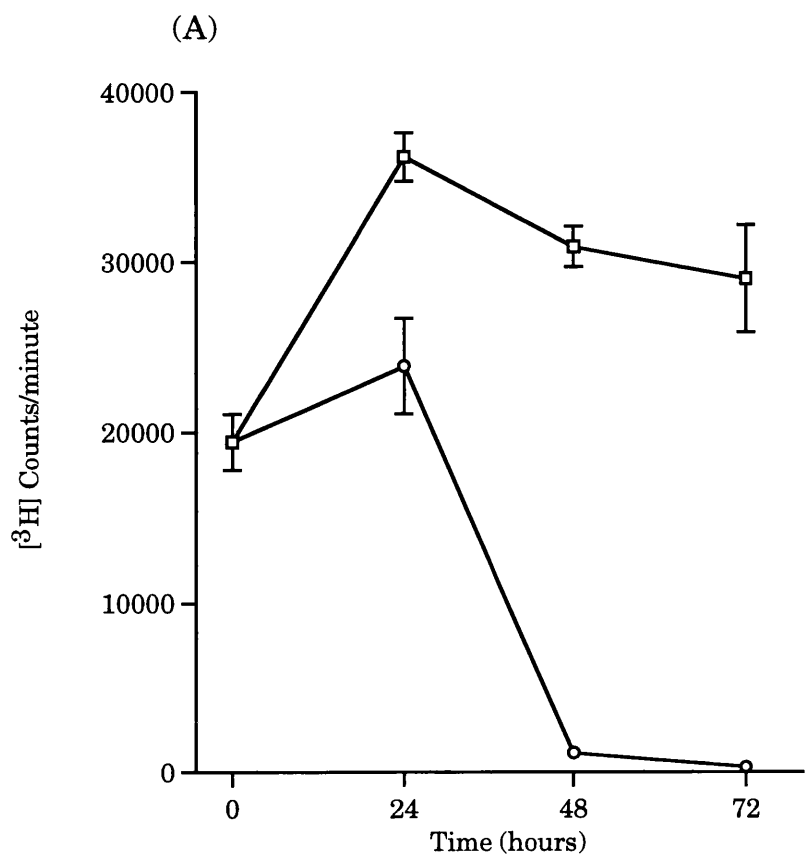
In the *Theileria* system, in addition to the effect of aphidicolin, recent experiments have analysed the influence of polypeptide synthesis on the induction of differentiation. It was found that elevation of general protein synthesis accompanies temperature and that these events are associated with the initiation of differentiation and the up-regulation of target gene expression (Shiels, *et al.*, 1997).

HL-60 differentiation by DMSO may also bring about changes in protein levels relative to DNA. To test this postulation, estimation of the rate of DNA and protein synthesis were carried out following incubation of cells with or without DMSO for 0, 24, 48 and 72 hours. Duplicate samples of cells were taken at daily time points and simultaneously assessed for both [<sup>3</sup>H] thymidine and [<sup>35</sup>S] methionine incorporation. As shown in Figure 4.9., the rate of DNA synthesis (Figure 4.9.A) was higher than protein synthesis (Figure 4.9.B) for both control and test cultures over the first 24 hours. The ratio of the increase in rate of protein relative to the increase of DNA synthesis was calculated as 0.522 for DMSO and 0.866 for the control. Between 24 and 48 hours however, the ratio of the rate of protein relative to DNA synthesis (16.5) was dramatically altered in the DMSO culture, due to a 23 fold reduction in the rate of DNA synthesis. A change was also found in the control culture, but this was small in comparison. This alteration in the rate of protein synthesis relative to DNA induced by DMSO was confirmed if the 48 hour values were compared directly to the control culture. Thus, although DMSO reduced the rate of protein synthesis by 8 fold, the rate of DNA synthesis was reduced by 30 fold. Further incubation to 72 hours exacerbated the change in the rate of protein synthesis to DNA for the DMSO culture (ratio of 2.7), while in the control culture the ratio returned to a value (0.89), which was similar to the ratio calculated at 24

**Figure 4.9.** Effect of DMSO on DNA and protein synthesis in HL-60 cells. Cultures were incubated for a total of 72 hours in the presence of either; (1) control cultures lacking DMSO or (2) cultures continuously exposed to 1.3% DMSO. Incorporation of radioactive label was estimated during the final 5 hours of incubation. Graph (A); the effect of DMSO on the incorporation of [<sup>3</sup>H] thymidine. Graph (B); the effect of DMSO on the incorporation of [<sup>35</sup>S] methionine.

Values represent the mean counts per minute per  $4 \times 10^5$  cells of 6 sample wells +/- the standard error.





—□— (1) control    —○— (2) DMSO

hours. Therefore, it was concluded from these results that DMSO significantly alters the rate of protein synthesis relative to DNA synthesis but, only following an initial 24 hours in the presence of the inducer.

#### 4.4. Discussion

Using monoclonal antibody 4H5 as a sensitive marker, experiments were undertaken to examine the process of DMSO induced differentiation in HL-60, and compare this to recently obtained data for *T. annulata*. A correlation was found between the number of NBT positive cells estimated at different time points and the number of cells showing positive reactivity with monoclonal antibody 4H5 (Figures 4.5.C and 4.6.). Thus, it was concluded that differentiation towards granulocytes was associated with a quantitative increase in the expression of the 160 kDa antigen. An apparent discrepancy between the numbers of NBT positive cells at day 5 and the number of 4H5 positive cells could be accounted for by the sensitivity of the two assays.

DMSO pulse experiments were used to examine the nature of commitment to full granulocyte differentiation. Generally, at DMSO incubation times below 24 hours there was no notable increase in the numbers of cells showing positive reactivity with monoclonal antibody 4H5 when compared to control cultures. At 12 and 18 hour incubation times, a cross back was observed between the populations of negative and plus-minus cells (Figures 4.5.B and 4.2.A). An initial decrease in negative cells and concomitant rise in plus-minus cells was subsequently followed by a recovery in numbers to a level comparable to that at day 0. This suggested that at pulse times below those required for commitment, low level expression of the g160 kDa antigen was reversed by removal of the inducer. When cultures were incubated in DMSO for greater than 24 hours a cross over was observed between the increasing positive population and the decreasing plus-minus and negative populations. These kinetics imply that in order for a major proportion of the plus-minus population to convert to positive cells, they

must be in the inducer for greater than 18 hours or they will revert to a negative population. However, once a cell has become fully positive it will not revert to a plus-minus or negative cell in the absence of inducer. These results support conclusions made previously Tarella, *et al.* (1982) on the commitment of HL-60 cells to granulocyte formation as assessed by NBT reduction, and show that the intermediate state is an important transition stage in the differentiation process. In addition, progress through the different stages during differentiation was associated with a quantitative increase in the expression of the 160 kDa antigen. This work has confirmed directly that, in both *T. annulata* (see 1.8.) and HL-60, the progression towards differentiation is linked to a reversible increase in the expression of target genes which are fully expressed upon reaching a commitment point. It would seem likely, therefore, that similarities will also be found in the basic molecular mechanisms controlling these events.

Affects on culture conditions caused by modulation of cell number has been found to influence differentiation potential in *T. annulata*. Cultures of the differentiation enhanced cell line seeded at low cell densities ( $1.4 \times 10^5$  cells  $\text{ml}^{-1}$ ) before incubation at  $41^\circ\text{C}$  were found to differentiate at a higher rate compared to cultures seeded at higher cell densities ( $2.8 \times 10^5$  cells  $\text{ml}^{-1}$ ) (Shiels and McKellar, unpublished data). Similar effects of culture conditions on HL-60 granulocyte differentiation were also found. Cultures which were left undiluted for 5 days and were shown to have higher levels of g160 kDa antigen than cells cultured normally. In one DMSO pulse experiment the results obtained from the shorter pulse times were unexpectedly variable. A possible explanation for this was that at these short pulse times the conditions in individual culture flasks effected the differentiation state of the cells. Comparison of the 6 hour, 18 hour and negative control cultures (Figure 4.3.1.) showed that a cross back between

negative and plus-minus cell populations took place in all three cultures. However, the results imply that conditions within each individual culture were different, as the cross-back occurred at different times. In addition, the proportion of negative (48%) and plus-minus (44%) cells in these experiments were almost equal at the start of the time course, whereas in other similar experiments there was a much higher proportion of negative cells, typically 60-80%, at day 0. This could indicate that the culture used to set up this particular experiment had been subjected to growth conditions which altered the differentiation status of the cells prior to the addition of DMSO thus, exacerbating the effect of the inducer. At longer pulse times there did not appear to be any similar effect of growth conditions, indicating that induction by DMSO at periods of longer than 24 hours was able to overcome any variations due to culture conditions. From these results it is possible to conclude that as growth conditions will vary during the 2-3 day period of routine culture, HL-60 cells in these cultures are constantly modulating between the negative and plus-minus state, with a few cells undergoing spontaneous terminal differentiation. Therefore, in agreement with the stoichiometric model proposed for merozoite differentiation in *T. annulata* (Shiels, *et al.*, 1994), the addition of DMSO would increase the probability of a spontaneous (random) differentiation event occurring.

In *T. annulata* changing the parameters of proliferation/growth directly by the addition of aphidicolin was shown to increase differentiation potential. Incubation of the differentiation enhanced cell line at either 41°C or 37°C for 24 hours in aphidicolin had no effect (Shiels, *et al.*, 1997). Extending the incubation times with aphidicolin beyond 24 hours was not possible as the drug significantly reduced the viability of the host cell. However, a constant low level rate of differentiation could be obtained if the differentiation enhanced cell line was pre-adapted to culture at 41°C by an 8 week

incubation at the elevated temperature. If aphidicolin was subsequently added to these pre-adapted cells for 24 hours at 41°C, a three fold increase in differentiation rate was observed, compared to similar control cultures without drug treatment. The addition of aphidicolin to HL-60 cultures was similarly shown to increase differentiation potential. Following drug treatment the number of cells expressing the g160 kDa antigen at both intermediate and high levels was increased (Figure 4.8.). In addition, following the removal of aphidicolin the number of positive cells continued to rise, suggesting that the effect of the drug treatment was to induce cells to progress through the commitment step of granulocyte differentiation.

Induction of HL-60 cells towards stationary phase growth or incubation with aphidicolin induced differentiation at a much lower level than that achieved by continuous exposure to DMSO. This could mean that only cells that were close to a quantitative threshold which determined commitment were in a position where these factors could influence differentiation status. For the rest of the cells, the time where conditions were optimal for reaching commitment may have been too short. Thus, a few cells were able to become fully positive, but the majority (56%) were only able to reach plus-minus before the removal of aphidicolin caused reversion to negative (Figure 4.8). Extending the incubation time with aphidicolin was not possible as this resulted in a significant loss of cell viability (Figure 4.7.). This was probably due to the induction of apoptosis before commitment could be reached, as recent studies have shown that aphidicolin can induce cell death in some cell types (Schimke, *et al.*, 1994; Waxman, *et al.*, 1992). For certain cell lines, commitment to terminal differentiation has been achieved following prolonged incubation with aphidicolin in a dose and time dependant manner (Cinatl, *et al.*, 1994; Griffin, *et al.*, 1982; Murate, *et al.*, 1990; Schwartz, *et al.*, 1995). This could reflect the inability of aphidicolin

to induce apoptosis in these cell types, or that the commitment to differentiate overrides the triggering of cell death pathways.

In *T. annulata* the effect of aphidicolin on parasite differentiation has been shown to be dependant on culture at 41°C. It appears that a second parameter associated with elevated temperature is involved in initiation and progression towards commitment. Evidence suggests that this second parameter is an elevation of protein synthesis, as following a short term incubation at 41°C a general increase in the rate of parasite polypeptide production has been found. In addition, inhibition of parasite protein production by oxytetracyclin has been shown to significantly reduce differentiation potential (Shiels, *et al.*, 1997). In HL-60 the results presented in Table 4.1. show that DNA synthesis was significantly inhibited in aphidicolin treated cells without a major decrease in the production of protein, supporting previous studies on the effect of the drug (Iliakis, *et al.*, 1982; Schimke, *et al.*, 1991). Thus, for both the parasite and for HL-60, potentiation of differentiation by aphidicolin is associated with an increase in general polypeptide synthesis relative to DNA synthesis. The ability of aphidicolin to induce differentiation in a number of eukaryotic cell types, including HL-60 (Cinatl, *et al.*, 1994; Griffin, *et al.*, 1982; Murate, *et al.*, 1990; Schwartz, *et al.*, 1995) and the observation that in *T. annulata* the reversible phase of differentiation to the merozoite is initiated by an elevation in the rate of protein synthesis (Shiels, personal communication), lead to the hypothesis that progression towards commitment involves an increase in the ratio of protein factors which regulate the gene expression of the next stage. Moreover, this hypothesis could also explain why many reagents which potentiate differentiation of HL-60 act at the level of inhibiting DNA synthesis or cell proliferation (Barrera, *et al.*, 1991; Cook, *et*

*al.*, 1990; Hui and Yung, 1993; Ling, *et al.*, 1991; Nagy, *et al.*, 1995; Taoka, *et al.*, 1990; Tarella, *et al.*, 1982; Waxman, *et al.*, 1992).

Classical inducers of HL-60 granulocyte differentiation, DMSO, dbcAMP and retinoic acid, could function by altering the ratio of protein production relative to DNA synthesis, as all these reagents have been shown to prolong the time that cells are held within the growth phases of the cell cycle (G1 and G2) (Brennan, *et al.*, 1991; Gezer, *et al.*, 1988; Horiguchi-Yamada and Yamada, 1993; Laskin, *et al.*, 1991). Experiments estimating the rate of protein synthesis relative to DNA following exposure to DMSO showed that over the first 24 hours the rate of DNA synthesis appeared to be greater than protein. However, between 24 and 48 hours the ratio was drastically reversed due to a major reduction in the rate of DNA synthesis. This change occurs after approximately 24 hours which, interestingly is the same period of incubation required to bring about significant granulocyte differentiation (Tarella, *et al.*, 1982) and g160 kDa antigen expression. These results indicate that while the initiation of differentiation could be due to an elevated rate of protein synthesis in *Theileria*, this is unlikely to be the case for HL-60 cells. Progression towards the commitment point however, may involve an alteration in the ratio of protein relative to DNA for both systems.

From the work presented in this chapter, the current studies on *T. annulata* and the work of others on HL-60 and related differentiation systems it is possible to formulate a simplistic model in which cells differentiate in a stochastic manner. This model is influenced by the model proposed by (Blau, 1992), where the relative concentration of regulators of gene expression play a critical role in the determination of the differentiated state. Under normal culture conditions the majority of cells maintain these



factors at a level below the threshold required for commitment to differentiation. However, within a population at any given time, individual cells could be nearer or further from the threshold, resulting in asynchrony within the population with respect to differentiation. Addition of an inducer would cause the shifting of the stoichiometry of regulators, such that progression towards a threshold occurs, in turn setting off a cascade of events leading to terminal differentiation. As many regulators of gene expression act in combination with other factors, small changes in the relative concentration of a single regulator could have large effects on gene expression. This change could be achieved, following alterations to the level of one factor relative to another, or a change in the ratio of factor relative to an autoregulatory DNA template. In *Theileria* this may be initiated by increases in polypeptide synthesis alone, but in HL-60, initiation is likely to be more complicated and involve the previously described immediate early events.

For both systems, however, extension of the G1/G2 phases of the cell cycle, could significantly influence progression towards commitment by the autoregulatory amplification of positive regulators, resulting in detectable changes to merozoite and granulocyte gene expression. Removal of the inducer before a particular concentration threshold was reached, however, would result in an increase in DNA templates by the re-establishment of cell proliferation. This in turn would lower factor production, leading to a reversal of the process, and a reduction in gene expression. A prediction of this model is that the random probability of a differentiation event occurring is linked to the concentration of key regulators of gene expression relative to autoregulatory DNA sequences. Clearly the aim of future research would be to test this model following characterisation of the factors which regulate merozoite and granulocyte target genes. A prerequisite for

this type of study is the isolation of gene clones encoding both target DNA sequences and regulatory peptide factors.

## **Chapter 5**

## Purification and cloning strategies of TaHBL20/125

### 5.1. Introduction

Previous chapters have described the characterisation of antigens which are recognised by monoclonal antibody 4H5 in *T. annulata* infected cells and in HL-60 cells. In HL-60, reactivity of the antibody for the g160 kDa antigen was found to be significantly increased following differentiation to granulocytes and this reactivity was used to further investigate parallels between differentiation to the merozoite in *T. annulata* and differentiation to granulocytes in HL-60. The identity and function of both these antigens remains unknown, but many of the unanswered questions discussed in the preceding chapters could be addressed by cloning the genes encoding one or both of these antigens. Two main strategies are available; a) transient expression of cloned genes transfected into mammalian cells followed by screening by antibody selection, b) direct screening of cDNA libraries with either antibody or with oligonucleotides.

Preliminary experiments were performed to determine the feasibility of screening a *Theileria* infected cell bacteriophage (phage) cDNA expression library using monoclonal antibody 4H5. The antibody failed to produce any signal during the screen, and no positive plaques were identified. This was not surprising, as recognition of TaHBL20/125 by this antibody is known to be susceptible to conditions which alter the physical conformation (Chapters 2 and 3). Expression of authentic, biologically active eukaryotic proteins from cloned DNA frequently requires post-translational modifications such as glycosylation, phosphorylation, specific proteolytic cleavage, accurate disulphide bond formation or oligomerisation. This problem is particularly severe for the expression of cell surface molecules (Sambrook, *et al.*, 1989).

As these processes are not performed by bacteria or phage systems, it was unlikely that the gene encoding the 4H5 antigen would be expressed in the correct physical conformation in a phage vector to allow recognition by the monoclonal antibody.

These problems can potentially be overcome by the expression of cloned genes in mammalian cells. A commonly used method for this technique is the transfection of cDNA libraries into the simian COS cell line (Gluzman, 1981). cDNA libraries are constructed in a plasmid vector which also contains elements from the eukaryotic simian virus 40 (SV40) as a source of a promoter and other elements such as, polyadenylation signals, splice acceptor and/or donor sequences and enhancers, which should allow accurate expression and post translational modification of an inserted cDNA. These plasmids are transfected into COS cells and clones expressing the desired molecules are identified by successive rounds of antibody panning (Seed and Aruffo, 1987). However, although transfected DNA may contain all the information required for post-translational modification of the desired polypeptide, there is no guarantee that it will perform normally in the transfected COS cell lines (Sambrook, *et al.*, 1989).

A second approach is the screening of cDNA libraries with oligonucleotides derived from the amino acid sequence of the antigen recognised by monoclonal antibody 4H5. A prerequisite for this is the isolation of adequate amounts of antigen for micropeptide sequencing. Sufficient amounts of antigen are most commonly isolated using immunoaffinity purification (Harlow and Lane, 1988). Results presented in Chapter 3 demonstrated that, recognition of g160 in HL-60 by monoclonal antibody 4H5 was significantly more sensitive to physical disruption when compared to TaHBL20/125. In addition, frequent attempts to immunoprecipitate the

molecule from HL-60 using this antibody had been unsuccessful. Therefore, if this method was to be undertaken in an attempt to clone the gene encoding the 4H5 antigen, it appeared that the best strategy was to undertake purification of the polypeptide from *Theileria* infected cells.

Time constraints did not permit cloning strategies to be undertaken to isolate the gene encoding TaHBL20/125 and g160. However, if the molecules recognised by monoclonal antibody 4H5 in TaHBL20 and in HL-60 are the bovine and human homologues of the same molecule, it might be possible to clone the g160 kDa polypeptide using nucleotide sequence data generated from the gene clone encoding TaHBL20/125. Even in the absence of the HL-60 gene, it was thought that characterisation of the nucleotide sequence in a *Theileria* cDNA clone could provide valuable information as to the possible identity and function of the antigen during HL-60 granulocytic differentiation.

Of the strategies discussed above, immunoaffinity purification of the TaHBL20/125 antigen was considered to be the most straight forward. The primary reason for this was that, it was unknown whether COS cells transfected with the cDNA encoding TaHBL20/125 or g160 would express the antigen in a conformation that would be recognised by monoclonal antibody 4H5.

#### **5.1.1. Aims**

The primary aim of work presented in this chapter was to use immunoaffinity chromatography to purify the TaHBL20/125 antigen for microprotein sequencing. Secondly, to use amino acid sequence data to design single stranded oligonucleotides from the obtained amino acid

sequence data and use these as probes to screen a cDNA library and identify clones representing the gene of interest. Thirdly, to characterise the gene of interest by sequence analysis.

## **5.2. Materials and Methods**

### **5.2.1. Coomassie staining of SDS-PAGE gels**

Following electrophoresis, gels were stained and fixed in a solution of 20% methanol, 10% acetic acid and 0.5% Coomassie Brilliant Blue R-250 in dH<sub>2</sub>O, for approximately 60 minutes and destained in the same solution lacking the Coomassie dye.

### **5.2.2. Purification of monoclonal antibody 4H5**

#### **5.2.2.1. Ammonium sulphate precipitation**

Monoclonal antibody supernatant (see 2.2.1.) was precipitated using ammonium sulphate to reduce the overall volume of material prior to further purification. An equal volume of saturated ammonium sulphate (50% cut) was added to 1.2 litres of monoclonal antibody 4H5 supernatant and incubated at 37°C with gentle agitation for 60 minutes. Precipitated antibody was pelleted by centrifugation at 10 000 g for 20 minutes and resuspended in 100 ml dH<sub>2</sub>O. Residual ammonium sulphate was removed by dialysis against 2 litres of PBS (Harlow and Lane, 1988).

#### **5.2.2.2. Protein A immunoaffinity chromatography**

Protein A affinity chromatography was used to purify monoclonal antibody 4H5. A 2 ml column of Protein-A-Sepharose 4 Fast Flow beads (Pharmacia) was pre-washed with 5 ml of 100 mM glycine, pH 3.0, followed by 20 ml of 100 mM Tris.HCl, pH 8.0. 100 ml of concentrated monoclonal antibody 4H5 (see 5.2.2.1.) was passed over the Protein A column at 4°C. The column was washed with 20 ml of 100 mM Tris.HCl, pH 8.0, followed by 20 ml of 10 mM Tris.HCl, pH 8.0, and bound antibody eluted with 10 ml of 100 mM glycine, pH 3.0. Fractions of 500 µl were collected in eppendorf tubes containing 50



µl of 1M Tris.HCl, pH 8.0 and the tubes gently mixed. Immunoglobulin containing fractions were identified by running 5 µl aliquots of each fraction on a 10% SDS-PAGE gel followed by staining with Coomassie blue (see 5.2.1.). A 5 µl sample of unpurified antibody was also run for comparison. Antibody containing fractions were pooled and the protein concentration estimated by the method of Bradford (see 2.2.6.1.).

#### **5.2.2.3. Biotinylation of monoclonal antibody 4H5**

150 µl of Sulfo-NHS-boitin, sulfosuccinimidobiotin (Pearce, 10 mg ml<sup>-1</sup> in 100 mM sodium borate buffer, pH 8.8) was added to 500 µl of purified monoclonal antibody 4H5 at a concentration of 1 mg ml<sup>-1</sup> in sodium borate buffer, and incubated at room temperature for 4 hours. 20 µl of 1 M ammonium chloride was then added and the sample incubated for a further 10 minutes at room temperature. Unbound biotin was removed by dialysis against several changes of PBS for 12 hours at 4°C. An indirect IFA (see 2.2.5.1.) was carried out on TaHBL20 and BL20 cells to verify the reactivity of the antibody.

#### **5.2.3. Immunoaffinity Purification of TaHBL20/125**

##### **5.2.3.1. Concanavalin A lectin affinity chromatography**

10 ml of concanavalin A (Con A) (*Canavalis ensiformis*, jack bean) sepharose 4B (Pharmacia) was pelleted by centrifugation at 5 000 g at 4°C for 5 minutes and washed twice with an equal volume of NP-40 lysis buffer (see 2.2.6.). The Con A sepharose was then incubated overnight at 4°C on a rotator with 4 ml of TaHBL20 NP-40 cell extract (see 2.2.6.). Unbound proteins were removed by 5 washes with NP-40 lysis buffer, and bound glycoproteins were eluted by incubation with 2 ml of 0.4 M methyl- $\alpha$ -D-mannopyranoside for 30 minutes on a rotator at room temperature. Con A

sepharose was pelleted by centrifugation at 5 000 g for 5 minutes and the supernatant retained. Eluted fractions were concentrated by centrifugation through a Centrocon filter with a cut off point of 30 kDa for 60 minutes at 5000 g. The protein concentration of eluates was determined by the method of Bradford (see 2.2.6.1.). 5 µg equivalents of eluted glycoprotein fraction and unpurified TaHBL20 extract were run on a 6% SDS-PAGE gel (see 2.2.7.) and immunoblotted with the anti-glycoprotein antiserum (see 2.2.4. and 2.2.8.).

### **5.2.3.2. Biotin/streptavidin 4H5 immunoaffinity column**

#### **Assessing the elution conditions for the immunoaffinity column**

50 µl of Con A fraction (see 5.2.3.1.) was incubated with 2 µl of purified, biotinylated 4H5 antibody for 90 minutes on ice. 30 µl of 6% ImmunoPure immobilised streptavidin (6% v/v in PBS, Pierce) was added, and incubated for a further 90 minutes on ice. The beads were pelleted by centrifugation at 10 000 g for 2 minutes, washed 4 times in PBS (see 2.2.5.), washed once in pre-elution buffer (See Table 5.1.), before incubation for 2 minutes with 25 µl of the appropriate elution buffer (see Table 5.1.). Following the elution step, the beads were pelleted by centrifugation, and the supernatant was immunoblotted and probed with the anti-glycoprotein antibody.

Once optimal elution conditions had been established in the preliminary experiments described above, immunoaffinity purification of TaHBL20/125 was under taken on a larger scale. 1 ml of Con A fraction (see 5.2.3.1.) was incubated overnight at 4°C with 40 µl of biotinylated purified monoclonal antibody 4H5. 5 ml of streptavidin beads were added and incubated for a further 2 hours at 4°C. The sample was then allowed to settle in a 10 ml chromatography column (Biorad) and washed with 20 ml of 10 mM

**Table 5.1.** Conditions used to elute TaHBL20/125 from biotininylated monoclonal antibody 4H5/streptavidin sepharose.

Elution conditions	Pre-elution buffer	Collection buffer
100 mM triethylamine, pH 11.5	10 mM phosphate, pH 8.0	1/10 volume 1 M Tris.HCl, pH 6.8
100 mM glycine, pH 2.5	10 mM phosphate, pH 6.8.	1/10 volume 1 M Tris.HCl, pH 8.0
100 mM glycine, pH 1.8	10 mM phosphate, pH 6.8	1/15 volume 1 M Tris.HCl ,pH 8.0
3.5 M MgCl <sub>2</sub> in 10 mM phosphate, pH 7.2	10 mM phosphate, pH 7.2	-----
2 M urea	PBS	-----
2 M guanidine. HCl	PBS	1/10 volume 1 M Tris.HCl, pH 8.0
8 M guanidine. HCl, pH 1.5	PBS	1/10 volume 1 M Tris.HCl, pH 8.0

phosphate buffer, pH 8.0. Bound antigen was eluted with 3 ml of 100 mM triethylamine, pH 11.5, and 200  $\mu$ l aliquots were collected in 1/10<sup>th</sup> volume of 1 M Tris.HCl, pH 8.0. To identify antigen containing fractions, 10  $\mu$ l aliquots of each fraction were loaded onto a 10% a SDS-PAGE gel (see 2.2.7.), Western blotted (see 2.2.8.) and probed with both the anti-glycoprotein antibody (see 2.2.4.) and with ExtraAvidin (Sigma) antibody. To obtain sufficient purified antigen for microprotein sequencing this procedure was repeated a further 8 times using the same immunoaffinity column, but with a fresh Con A fraction each time. All fractions containing purified antigen were pooled (approximately 16 ml) and dried using a Savant speedivac concentrator in 1 ml aliquots.

#### **5.2.4. Protein sequencing**

##### **5.2.4.1. SDS-PAGE**

SDS-PAGE for the preparation of proteins for microsequencing was carried out according to the method of Dunbar and Wilson (1994) using the Biorad Protean II gel electrophoresis system. A 10 % slab gel composed of 4.17 ml dH<sub>2</sub>O, 3.325 ml acrylamide mix (30% acrylamide (99% pure, Fluka), 0.8% piperazine diacrylamide (PDA, Bio Rad)) and 2.5 ml lower buffer (0.375 M Tris. HCl, pH of 8.8, 2% w/v SDS). The solution was degassed for 5 minutes before polymerisation was initiated by the addition of 2.5  $\mu$ l TEMED and 37.5  $\mu$ l of freshly made 10% APS. After pouring, the gel was overlaid with water saturated 2-butanol and allowed to set for approximately 60 minutes. Once the resolving gel had polymerised, the overlay was poured off and the top of the gel rinsed with dH<sub>2</sub>O. 5 ml of a 5% stack gel was then poured on top of the resolving gel. The stack gel was composed of 2.925 ml dH<sub>2</sub>O, 0.825 ml acrylamide mix and 1.25 ml lower buffer. The solution was degassed for 5 minutes before polymerisation was initiated by the addition

of 5  $\mu$ l TEMED and 37.5  $\mu$ l 10% APS. Upper buffer (0.125 M Tris. HCl, pH 6.8, 2% w/v SDS) containing 10 mM filtered glutathione was added to the upper reservoir of the electrophoresis tank, while lower buffer (see above) was added to the lower reservoir. 10  $\mu$ l of sample buffer (10% v/v glycerol, 2.5% v/v 2-mercaptoethanol, 2% w/v SDS, 0.0625 M Tris.HCl, pH 6.8 and 0.002% w/v bromophenol blue) was loaded into one well and the gel was pre-run at 6 mM until the dye reached the bottom of the gel. All buffers were then discarded and replaced with running buffer (see 2.2.7.). In addition, 1  $\mu$ l ml<sup>-1</sup> of 100 mM sodium thioglycolate was added to the upper reservoir only. 5 aliquots of purified, lyophilised TaHBL20/125 antigen (see 5.2.3.2.) were resuspended in a total of 60  $\mu$ l of dH<sub>2</sub>O and diluted 1:1 in sample buffer (see above). The sample was then heated to 100°C for 5 minutes and loaded onto the pre-run gel. Electrophoresis was carried out at a constant current of 25 mA until the dye front reached the bottom of the gel. The molecular masses of proteins were estimated by reference to high molecular mass standard polypeptides (29-205 kDa, Sigma)

#### **5.2.4.2. Western blotting**

Proteins separated for sequencing were transferred onto pre wetted membrane (Pro Blott, Applied Biosystems) in 3-[cyclohexylamino]-1 propanesulfonic acid (CAPS) buffer (10 mM CAPS, pH 11.5, 10% v/v methanol), using a Bio-Rad Trans blot cell at a constant of 50 V (170-100 mA) for 2 hours at room temperature. The blot was removed, rinsed in dH<sub>2</sub>O for 5 minutes and then stained with Amido black (0.1% in 40% methanol, 1 % acetic acid) for 1 minute. The blot was destained in dH<sub>2</sub>O and allowed to air dry at room temperature for 3-4 hours. The band of interest was excised from the blotting membrane and stored at -20°C. Microprotein sequencing was carried out by Mr B Dunbar of the amino acid

sequencing facility at University of Aberdeen, according to the method of Matsudaira (1987).

#### **5.2.5. Synthesis and end labelling of oligonucleotides.**

4 degenerate oligonucleotide pools were synthesised, 4H5POA1-4 (Molecular Biology laboratory, University of Strathclyde) from amino acid sequence data generated in 5.2.4.2. A stretch of amino acids was chosen to give the longest oligonucleotides with the least degeneracy. The oligonucleotides were synthesised to give all possible combinations of codon preference for each amino acid and split into 4 pools containing 71 of the 284 total possible combinations (Figure 5.8.). The oligonucleotides were end labelled using [ $\gamma^{32}\text{P}$ ] and  $\text{T}_4$  polynucleotide kinase (PNK). 200 ng of each oligonucleotide was added to 2  $\mu\text{l}$  of 10 x PNK buffer (700 mM Tris.HCl, pH 7.8, 100 mM magnesium chloride, 50 mM dithiothreitol, Promega), 4  $\mu\text{l}$  (60 pmol) of [ $\gamma^{32}\text{P}$ ] ATP (>7000 Ci/mmol, ICN), and the volume made up to a total of 20  $\mu\text{l}$  with  $\text{dH}_2\text{O}$ . 0.5  $\mu\text{l}$  (5 units) of  $\text{T}_4$  PNK was added and the reaction incubated at 37°C for 20 minutes, at which point a further 0.5  $\mu\text{l}$  of PNK was added and the reaction incubated as before. Unincorporated [ $\gamma^{32}\text{P}$ ] ATP was removed by size exclusion chromatography through a NucTrap Probe purification column (Stratagene). The volume was made up to 70  $\mu\text{l}$  with STE (100 mM sodium chloride, 20 mM Tris.HCl, pH 7.5, 10 mM EDTA) and the reaction passed down a previously equilibrated column (70  $\mu\text{l}$  STE) using a syringe. Labelled oligonucleotide was recovered from the column and used directly in hybridisation experiments.

#### **5.2.6. Oligonucleotide screening of bacteriophage cDNA libraries**

A  $\lambda$  ZAP cDNA library constructed by Dr J. Kinnaird (WUMP, University of Glasgow) from a cloned *T. annulata* (Ankara) macroschizont infected cell line (D7) grown at 37°C was screened with the radiolabelled oligonucleotide

probes. *Eschericia coli* strain Y1090 (Promega) was the host bacterium used to plate out the  $\lambda$  ZAP D7/37 library. The bacteria were streaked out onto Luria-Bertani (LB) plates (1% bactotryptone, 0.5% yeast extract, 1% sodium chloride, 1.5% bactoagar) containing 100  $\mu\text{g ml}^{-1}$  ampicillin and incubated overnight at 37°C. The following day overnight cultures were prepared by picking one colony into 10 ml of LB/ampicillin media (as LB plates, minus bactoagar) containing 0.2 % maltose and 10 mM magnesium sulphate, and growing overnight on an orbital shaker at 37°C. 50 ml of supplemented LB medium was inoculated with 0.5 ml of overnight culture, and incubated in an orbital shaker at 37°C until an absorbance at 600 nm of 0.5 was obtained (corresponding to a cell density of  $2.5 \times 10^8$  cells  $\text{ml}^{-1}$ ). The cells were pelleted by centrifugation at 800 g for 5 minutes at 4°C followed by re-suspension in 5 ml of 10 mM magnesium sulphate. Cells were stored at 4°C for up to 2 days.

To screen the  $\lambda$  Zap D7/37 library,  $7.5 \times 10^4$  plaque forming units (pfu) in SM buffer (50 mM Tris.HCl, pH 7.5, 10 mM magnesium sulphate, 100 mM sodium chloride, 0.01% w/v gelatine) were absorbed onto 800  $\mu\text{l}$  of Y1090 cells in 10 mM magnesium chloride by incubation at room temperature for 15 minutes. 50 ml of top agarose (LB broth containing 0.7% low EEO agarose (Sigma) at approximately 45°C was added to the cells, and the sample was poured onto a 200 mm x 200 mm LB/ampicillin plate. Once set, the plate was incubated at 37°C overnight and chilled at 4°C for 1 hour. Plaque lifts were taken by laying a large nitrocellulose filter on top of the plate for 1 minute, and marking the position on the plate with a needle. The filter was immersed in a shallow tray (DNA side up) containing Whatman 3MM filter paper soaked in denaturing solution ( 0.5 M NaOH, 1.5 M NaCl) for 5 minutes followed by immersion for 2 x 3 minutes in neutralising solution (1.5 M NaCl, 0.5 M Tris.HCl, pH 7.4), and rinsing in 2

x SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0). To fix the DNA, the filter was baked at 80°C for 2 hours.

Prior to hybridisation the filters were first incubated with 15 ml of PIPES hybridisation buffer (100 mM NaCl, 50 mM PIPES, pH 6.8, 50 mM sodium phosphate buffer, 1 mM EDTA, 5 % SDS) for 60 minutes. This buffer was discarded and approximately 20 ng [ $\gamma^{32}\text{P}$ ] labelled oligonucleotide probe (see 5.2.5.) added in a further 15 ml of PIPES hybridisation buffer. The filter was hybridised at 37°C for 24 hours, washed 3 x 15 minutes in 6 x SSC, 0.5 % SDS at 37°C and exposed to autoradiography film (Kodak X-O mat AR) at -70°C. Autoradiographs were developed as described in 2.2.8. Positive plaques were identified and picked into SM buffer containing 20  $\mu\text{l}$  of chloroform and stored at 4°C. In addition, one negative plaque was also picked from each oligonucleotide pool to serve as a negative control in subsequent rounds of screening. To rescreen putative positive plaques, 3 000 pfu were absorbed onto 500  $\mu\text{l}$  of bacteria in magnesium sulphate (prepared as above) and plated onto 90 mm diameter LB/ampicillin plates in 2.5 ml of top agarose.

#### **5.2.7. *In vivo* excision**

200  $\mu\text{l}$  of *E. coli* XL1-Blue MRF' bacterial cells (Stratagene) incubated in an orbital shaker at 37°C until an absorbance at 600 nm of 0.1 was obtained, were combined with 200  $\mu\text{l}$  of  $\lambda\text{ZAP}$  phage stock (approximately  $1 \times 10^5$  phage particles) representing clones isolated from 5.2.6. and 1  $\mu\text{l}$  of R408 helper phage (approximately  $1 \times 10^6$  pfu  $\text{ml}^{-1}$ ). A negative control containing XL1-Blue cells and helper phage alone was also set up. The mixtures were incubated at 37°C for 15 minutes, followed by the addition of 5 ml of 2 x YT media (1% NaCl, 1% yeast extract, 1.6% bacto-tryptone) and further incubation at 37°C with shaking for 3 hours. The tubes were then



heated to 70°C for 20 minutes and centrifuged for 5 minutes at 4 000 g. The supernatants were decanted into sterile tubes and stored at 4°C for up to 2 months. To plate the rescued phagemid, 200 µl of stock phagemid was incubated with 200 µl of XL1-Blue bacterial cells with an absorbance at 600 nm of 0.1 (see above) for 15 minutes at 37°C and 25 µl plated onto LB/ampicillin plates (50 µg ml<sup>-1</sup>), followed by incubation overnight at 37°C. This was also carried out with 20 µl of a 10<sup>-2</sup> dilution of the stock phagemid. Colonies were streaked onto fresh LB/ampicillin plates the next day. Glycerol stocks were made of selected colonies. The cells were pelleted from a 10 ml overnight culture by centrifugation at 800 g for 5 minutes at 4°C, re-suspended in 3 ml of LB medium containing 10 % glycerol and stored at -70°C.

#### **5.2.8. Preparation of DNA**

1 to 3 ml of cells from an overnight bacterial cell culture were pelleted by centrifugation at 15 800 g for 5 minutes. The cell pellet was taken up in 200 µl of resuspension solution (50 mM Tris. HCl, pH 7.5, 10 mM EDTA, 100 mg ml<sup>-1</sup> RNase A), lysed by the addition of 200 µl lysis solution (200 mM NaOH, 1 % SDS) and neutralised by the addition of 200 µl 2.55 M potassium acetate, pH 4.8. Samples were centrifuged at 15 800 g for 5 minutes and the supernatant decanted into fresh tubes. DNA was precipitated by the addition of 2 volumes of ethanol and incubation at -20°C for 30 minutes. DNA was pelleted by centrifugation, and washed with 75% ethanol before drying for approximately 5 minutes under vacuum. DNA pellets were resuspended in sterile dH<sub>2</sub>O and stored at -20°C.

#### **5.2.9. Agarose gel electrophoresis**

DNA was separated by agarose gel electrophoresis as described by Sambrook, *et al.* (1989). 0.7 to 1% agarose gels were prepared by melting

agarose in an appropriate volume of either TBE (45 mM Tris-borate, 1 mM EDTA) or TAE (40 mM Tris-acetate, 1 mM EDTA). When cooled to hand temperature, ethidium bromide was added to a final concentration of 0.5  $\mu\text{g ml}^{-1}$ , the agarose poured into casting frame and the gel allowed to set at room temperature. When DNA fragments were to be excised from the gel, low melting point agarose (Sigma) was used and the gels were cast and run at 4°C. Samples were diluted 1:1 in 6 x sample buffer (0.2% bromophenol blue, 0.25% xylene cyanol, 40% (w/v) sucrose) and electrophoresed at 60 V in either TAE or TBE buffer until the xylene cyanol dye front was approximately two thirds down the gel. The size of DNA fragments was estimated by comparison to the 1 kb marker ladder (Gibco BRL), which ranged from 12 kb to 75 bp. Bands were visualised by exposure to short wave ultraviolet light (366 nm).

#### **5.2.10. Restriction of DNA**

0.2 to 1  $\mu\text{g}$  of DNA made up to 18  $\mu\text{l}$  total volume with  $\text{dH}_2\text{O}$ , was mixed with 2  $\mu\text{l}$  of the appropriate 10 x REACT buffer (Gibco BRL) and 1 to 2 units of restriction enzyme (Gibco BRL), followed by incubation at 37°C for 60 minutes in a water bath. On completion of reactions, restriction enzymes were heat inactivated at 65°C for 10 minutes.

#### **5.2.11. Mapping of $\lambda$ Zap clones by restriction digest and Southern blotting**

Recombinant DNA from the  $\lambda$  Zap clones was purified (see 5.2.8.), digested with a variety of restriction enzymes (see 5.2.10.) and run on a 1% agarose gel (see 5.2.9.). Following electrophoresis the gel was denatured in a solution of 1.5 M NaCl, 0.5 M NaOH for 45 minutes, neutralised in 2 M NaCl, 0.5 M Tris-HCl, pH 7.4, for 30 minutes and equilibrated in transfer buffer (50 mM phosphate buffer, 25 mM  $\text{NaH}_2\text{PO}_4$  pH 5.5 with 25 mM

Na<sub>2</sub>HPO<sub>4</sub>) for 30 minutes. The DNA was transferred to nylon membrane filters (Hybond-N, Amersham) by capillary action for at least 18 hours in transfer buffer. The DNA was fixed to the membrane by exposure to 150 Joules short wave ultra-violet radiation using a GS Gene Linker (Biorad). Prior to hybridisation, Southern blots were first incubated with 15 ml of PIPES hybridisation buffer (50 mM Pipes pH 6.8, 100 mM NaCl, 50 mM sodium phosphate buffer, 1 mM EDTA, 5% SDS) at 37°C for 60 minutes. This buffer was discarded and labelled oligonucleotide probes (see 5.2.5.) added in a further 15 ml of PIPES hybridisation buffer. Hybridisation was carried out overnight at 37°C. The filters were washed three times with 3 x SSC, 3% SDS at 37°C and exposed to autoradiography film at -70°C (see 5.2.6.)

#### **5.2.12. Sub-cloning of $\lambda$ Zap cDNA clones**

The small fragments identified in 5.2.11. as containing the DNA binding sequences for the degenerate oligonucleotides were subcloned into pBluescript SK (+/-) to facilitate further sequencing. DNA was first digested with the appropriate enzyme (see 5.2.11.). The Recessed ends of restriction fragments generated by digestion with *Msp* I (clones 2C1A and 4A1) or *Rsa* I (clone 1C1A) were filled in prior to electrophoresis by adding 1  $\mu$ l each of dATP, dCTP, dGTP and dTTP (0.5 mM, Boehringer Mannheim) and 2 units Klenow DNA polymerase (Boehringer Mannheim) to the restricted DNA and incubating for 60 minutes at 37°C. Restriction digests were then subjected to electrophoresis through a 1% low melting point TAE agarose gel. Bands of interest were excised and purified using QIAquick gel extraction kit, according to manufacturers instructions (Quiagen).

The pBluescript SK (+/-) vector was prepared for cloning by digestion with either *Sma* I for blunt ended cloning or with *Bam*HI for the cloning of the

*Sau*3A1 fragment of 4E1A (see 5.2.10.). To prevent vector-vector ligations the cut vector was then de-phosphorylated with calf intestinal alkaline phosphatase (CIAP). 20 µg of restricted vector was extracted once with Tris saturated phenol and once with Tris saturated phenol:chloroform (1:1), ethanol precipitated (see 5.2.8.) and resuspended in 45 µl of dH<sub>2</sub>O. 5 µl of 5 x CIAP buffer (500 mM Tris.HCl, pH 8.5, 1 mM EDTA) and 30 units of CIAP (Gibco BRL) was then added and the mixture incubated at 37°C for 60 minutes. To inhibit the CIAP, proteinase K was added to a final concentration of 100 µg ml<sup>-1</sup> and the mixture incubated at 56°C for 30 minutes. The mixture was heated to 75°C for 10 minutes followed by phenol extraction and ethanol precipitation (see 5.2.8.).

Ligations were set up as follows; Cohesive end ligations were performed using 0.2 µg of insert DNA and 0.2 µg of cut vector DNA, while blunt ended ligations using 0.6 µg insert DNA and 0.2 µg vector DNA were used. The DNA was made up to a total volume of 18 µl with dH<sub>2</sub>O and 2 µl of 10 x ligation buffer (500 mM Tris.HCl, pH 7.8, 100 mM dithiothreitol, 1 mM ATP, 25 µg ml<sup>-1</sup> bovine serum albumin) and 100 units of T<sub>4</sub> DNA ligase (400 units ml<sup>-1</sup>, New England Biolabs) was added. The ligation reactions were incubated at 16°C overnight.

### **5.2.13. Preparation of competent cells and transformation**

1 ml of an overnight culture (see 5.2.6.) of *E. coli* XL1-Blue was mixed with 100 ml of TYM broth (20% tryptone, 0.5% yeast extract, 100 mM NaCl, 10 mM MgSO<sub>4</sub>.7H<sub>2</sub>O) and incubated at 37°C with shaking until an absorbance at 600 nm of 0.5-0.9 was reached. The cells were diluted to 500 ml with TYM broth and incubated further, until an absorbance at 600 nm of 0.6 was reached. Cells were rapidly cooled in ice-water and then centrifuged at 4 200 g for 15 minutes at 4°C. The cell pellet was re-suspended in 100 ml of

ice-cold transformation buffer I (TfBI, 30 mM potassium acetate, 50 mM magnesium chloride, 100 mM potassium chloride, 10 mM calcium chloride, 15% (v/v) glycerol) and centrifuged at 4 200 g for 8 minutes at 4°C. The cells were then re-suspended in 20 ml of ice-cold TfBII (10 mM MOPS, pH 7.0, 75 mM calcium chloride, 10 mM potassium chloride, 15% (v/v) glycerol). 100 µl aliquots were rapidly frozen on dry ice and stored at -70°C. For transformation, aliquots were kept at room temperature until just beginning to thaw, before being placed on ice. DNA was added, and the cells left on ice for 30 minutes followed by a heat shock of 42°C for 2 minutes. 600 µl of LB broth was added and the cells incubated at 37°C for 90 minutes to allow the expression of antibiotic resistance markers encoded by the plasmid. Cells were then plated out onto LB/ampicillin plates to which 40 µl of X-gal (5-bromo-4 chloro 3 indolyl β-D galactosidase, 20 mg ml<sup>-1</sup> in dimethylformamide, Gibco BRL) and 4 µl of IPTG (isopropylthio-β-D galactosidase, 10 mg ml<sup>-1</sup>, Gibco BRL) had previously been spread on the surface and allowed to dry. Plates were incubated overnight at 37°C, and white recombinant colonies were picked and streaked onto fresh LB/ampicillin plates the next day.

#### **5.2.14. Automated DNA sequencing**

DNA fragments generated in sequencing reactions were labelled via extension from a sequencing priming oligonucleotide covalently coupled to an infrared fluorophore (laser dye IRD41). Excitation of labelled fragments by a laser diode emitting at 785 nm allowed the collection of the DNA sequence image during electrophoresis through acrylamide by the scanning of the gel with a microscope/detector (Middendorf, *et al.*, 1992). Samples for sequence analysis were generated by combining approximately 500 ng of DNA (see 5.2.8.), 2 pmol of labelled T7 or T3 primer, 2.5 µl of 10 x sequencing buffer (0.5 M Tris.HCl, pH 9.3, 25 mM magnesium chloride), 1

μl of BioPro thermostable DNA polymerase (Bioline) and dH<sub>2</sub>O to a final volume of 17 μl. Into each of 4 thermocycler tubes was placed 2 μl of each dideoxy termination mix (180 μM 7-deaza-dGTP, 50 mM NaCl; 180 μM dATP, 50 mM NaCl; 180 μM dCTP, 50 mM or 180 μM dTTP, 50 mM NaCl) together with 4 μl of the template/primer/enzyme mix. 30 μl of mineral oil was placed on the top of each reaction mixture and the tubes incubated in a thermocycler for the following cycles: initially 95°C for 5 minutes; followed by; 95°C for 30 seconds (denaturing step), 60°C for 30 seconds (annealing step) and 70°C for 1 minute (elongation step). A total of 30 cycles were performed and the reactions maintained at 4°C. After the cycling program was completed, 4 μl of stop solution (95% formamide, 20 mM EDTA, pH 7.6, 0.1% bromophenol blue, 0.1% xylene cyanol FF) was added to each reaction mix. Samples were denatured by heating to 95°C for 3 minutes prior to loading onto a 6% acrylamide gel. Gels were prepared by dissolving 21 g of urea in 6 ml of LongRanger™ gel mix (50%, FMC), 6 ml of 10 x TBE (890 mM Tris base, 890 mM boric acid, 20 mM EDTA) and dH<sub>2</sub>O to a total volume of 50 ml. The acrylamide solution was then degassed for 15 minutes before polymerisation was initiated by the addition of 25 μl TEMED and 250 μl freshly made 10% APS. The gel was cast using 41 cm x 25 cm x 0.5 cm LI-COR gel plates and left for approximately 60 minutes to set. Assembly of the LI-COR 4000 DNA sequencer apparatus was carried out according to the manufactures instructions. The upper and lower tanks were filled with 1 x TBE, and the gel was pre-run for approximately 30 minutes in order to attain a running temperature of 50°C. 2 μl of each sample was loaded, and electrophoresis was carried out for approximately 6.5 hours at 1500 V, 35 mA. Sequence information was collected directly by computer.

## **5.3. Results**

### **5.3.1. Immunoaffinity purification of the TaHBL20/125 antigen**

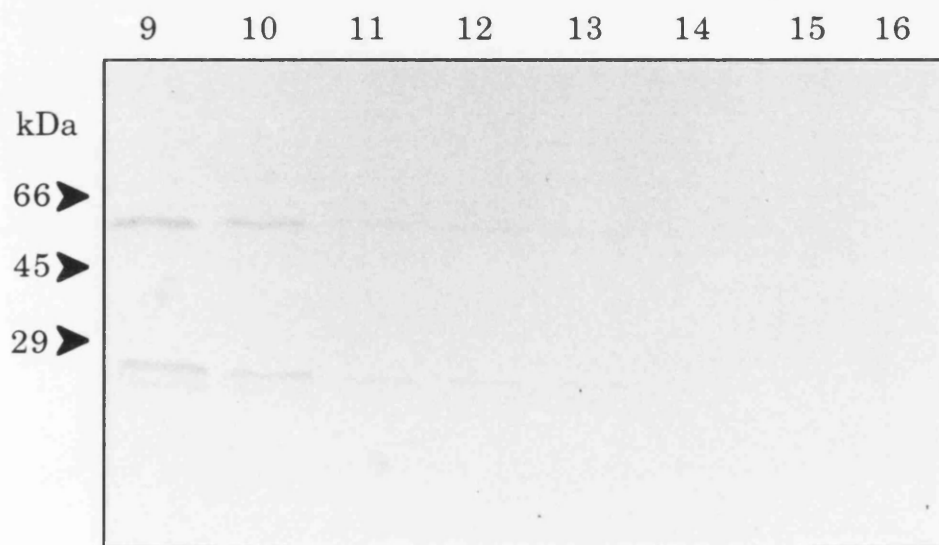
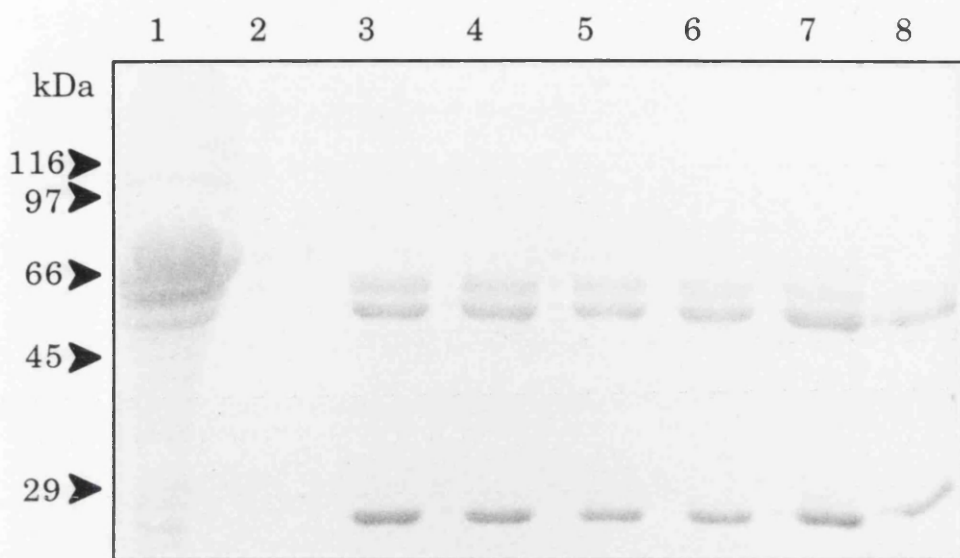
To obtain sequence data which would allow the isolation the gene encoding TaHBL20/125, the polypeptide had to be purified in sufficient quantity for microprotein sequencing. Often polypeptides of interest can be purified directly by excision from stained SDS-PAGE gels. However, staining acrylamide gels with Coomassie blue or with Periodic acid-Schiff (PAS), a method which specifically detects glycoconjugates, failed to clearly identify a polypeptide corresponding to TaHBL20/125 (data not shown). This could mean that the antigen was present at a low level in cell extracts, was disguised by another polypeptide of similar molecular mass or was not detected by these staining methods. To both purify and concentrate TaHBL20/125, immunoaffinity purification using monoclonal antibody 4H5 was undertaken. To generate an immunoaffinity column it was necessary to first purify the antibody from hybridoma supernatants. The overall volume of hybridoma supernatant was initially reduced by ammonium sulphate precipitation (see 5.2.2.1.), then the gamma globulin (IgG) enriched sulphate cut fraction was passed over a protein A sepharose column (see 5.2.2.2.). Protein A specifically binds to the Fc region of IgG subclasses of antibody, binding to and subsequent elution from the column resulted in the isolation of pure monoclonal antibody. Antibody containing fractions were identified on Coomassie stained SDS-PAGE gels and pooled. Bands corresponding to the heavy (55 kDa) and light (25 kDa) immunoglobulin chains were visible in fractions 3 to 12. An absence of any other polypeptides confirmed that the antibody had been efficiently purified (Figure 5.1.).

**Figure 5.1.** Coomassie-stained SDS-PAGE gel of successive fractions of purified monoclonal antibody 4H5 eluted from protein A sepharose column using 100 mM glycine.

Lane 1                    Monoclonal antibody 4H5 prior to passage over protein A  
                                 column

Lanes 2 to 16    successive elution fractions





TaHBL20/125 was thought to be present at a low level in cell extracts (see above) and was known to be glycosylated. To increase the concentration of antigen, therefore, and to act as a crude purification step, TaHBL20 extracts were initially enriched for glycoproteins by lectin chromatography. NP-40 extracts were passed over Con A sepharose, and bound glycoproteins eluted with the competing sugar, methyl-x-d-mannopyranoside. Enrichment of the extracts was assessed by immunoblotting with the anti-glycoprotein antiserum. Comparison of the lectin eluted fraction (Figure 5.2. lane 3) with normal NP-40 extracts of TaHBL20 (Figure 5.2. lane 1) showed significant enrichment of TaHBL20/125.

To purify the TaHBL20/125 antigen further, enriched lectin eluted fractions were passed over a biotinylated monoclonal antibody 4H5/streptavidin sepharose immunoaffinity column. Optimal elution conditions were established in a preliminary experiment. The level of TaHBL20/125 obtained using a variety of reagents was assessed by immunoblotting with the anti-glycoprotein antiserum (see 5.2.3.2.). Comparison of the different conditions showed that 100 mM triethylamine pH 11.5 gave the best elution profile (data not shown), and therefore was used, in the subsequent larger scale antigen purifications.

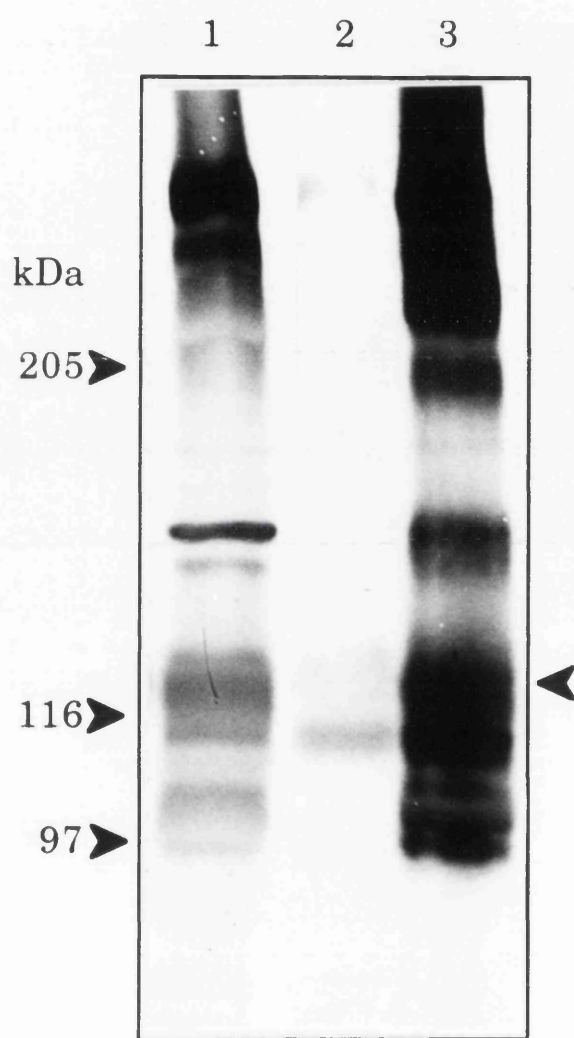
To increase the amount of TaHBL20/125 isolated using the monoclonal antibody 4H5 immunoaffinity column, greater volumes of Con A lectin eluate were passed over a larger biotinylated monoclonal antibody 4H5/streptavidin immunoaffinity column (see 5.2.3.2.). Fractions containing TaHBL20/125 were identified as before by immunoblotting with the anti-glycoprotein antiserum (Figure 5.3.). Simultaneous immunoblotting of eluted fractions with ExtrAvidin anti-streptavidin antibody also allowed an estimation of the effect of elution conditions on the

**Figure 5.2.** Analysis of a TaHBL20 extract before and after ConA lectin chromatography by immunoblotting and probing with anti-glycoprotein antibody. Arrow indicates position of TaHBL20/125.

Lane 1 TaHBL20 NP-40 extract

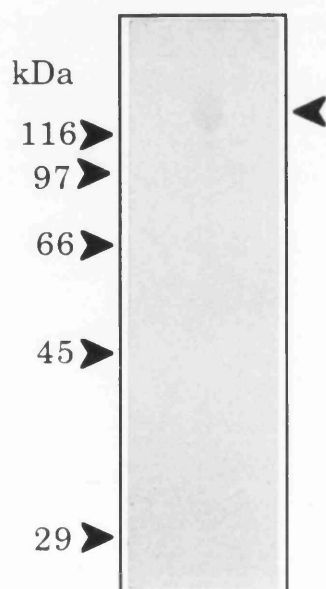
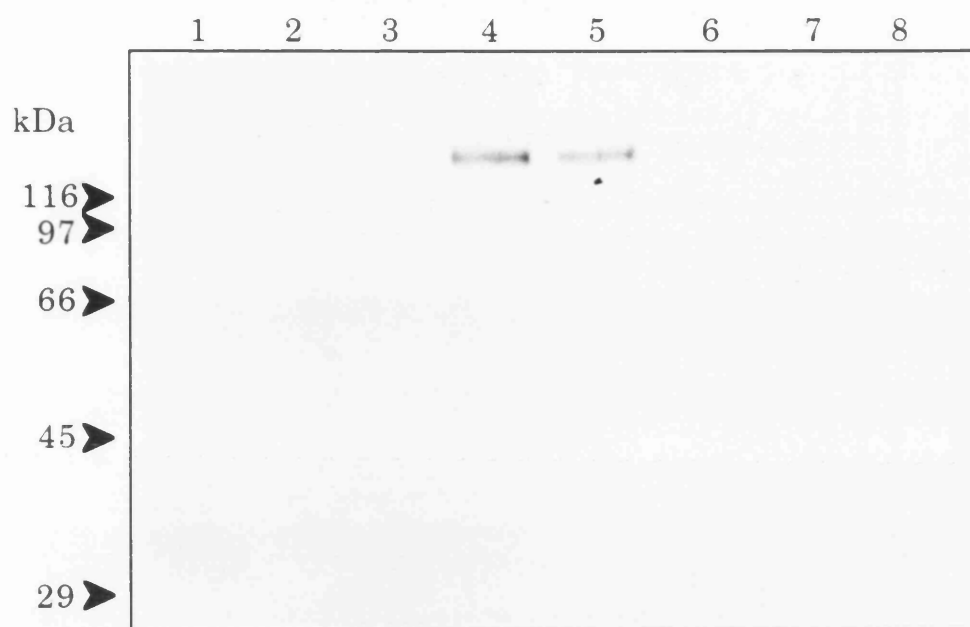
Lane 2 Unbound fraction

Lane 3 methyl-x-d-mannopyranoside eluted fraction



**Figure 5.3.** Elution profile of the TaHBL20/125 antigen from biotinylated monoclonal antibody 4H5 /streptavidin sepharose immunoaffinity column. 20 µl of each elution sample was immunoblotted with both the anti-glycoprotein antiserum and ExtraAvidin antibody. Lanes 1 to 8 refer to successive eluted fractions.

**Figure 5.4.** Coomassie-stained SDS-PAGE gel of a concentrated sample eluted from the biotinylated monoclonal antibody 4H5/streptavidin immunoaffinity column. Location of the TaHBL20/125 antigen is indicated by an arrow



coupling of the antibody to the sepharose column. No bands corresponding to the antibody were seen in the eluted fractions (Figure 5.3.). This indicated that significant amounts of antibody were not lost from the column and it could therefore, be used for repeated rounds of antigen isolation. The immunoaffinity purification procedure was repeated using the same column and fresh Con A enriched fractions a total of 9 times. After this time, the column failed to yield any further significant amounts of antigen. The antigen containing fractions from each round of purification were identified and pooled together. To increase the concentration of TaBL20/125 still further, the pooled fractions were lyophilised and re-suspended in reduced volume of dH<sub>2</sub>O. A small sample of the purified antigen was run on an acrylamide gel and stained with Coomassie blue to assess purity (Figure 5.4.). A larger sample was then Western blotted in preparation for microprotein sequencing (see 5.2.4.).

### **5.3.2. Amino acid sequencing of the TaHBL20/125 antigen**

The N terminal region of TaHBL20/125 was sequenced (section 5.2.4.) and the amino acid sequence obtained presented in Table 5.2. Comparison of the peptide with sequences in the Swiss Prot data base showed that the N-terminal of the TaHBL20/125 antigen had a 64% identity to the human ICAM-1 (Staunton, *et al.*, 1988) and, taking conservative substitutions into account (Sambrook, *et al.*, 1989) it was found that there was 82% similarity with this molecule (Figure 5.5.). The amino acid sequence of TaHBL20/125 was also compared to the ICAM-1 N-terminal amino acid sequence of mouse (Ballantyne, *et al.*, 1989), rat (Kita, *et al.*, 1992) and dog (Manning, *et al.*, 1995) ICAM-1 (Figure 5.6). A comparison of these sequences with human ICAM-1 was also carried out (Table 5.3.). This analysis revealed that there was between 42% and 52% identity with human ICAM-1 across the complete sequence, while lower identity (between 36% and 45% ) was

**Table 5.2.** Amino acid sequence of the N-terminal region of the TaHBL20/125 antigen. A (?) indicates an amino acid where the residue was present in a concentration too small to be distinguished.

Amino acid number	Amino acid	Amino acid abbreviation	Amino acid symbol	Number of codons
1	Glycine	Gly	G	4
2	Isoleucine	Ile	I	3
3	Serine	Ser	S	6
4	Isoleucine	Ile	I	3
5	Alanine	Ala	A	4
6	Proline	Pro	P	4
7	Serine	Ser	S	6
8	Lysine	Lys	K	2
9	Alanine	Ala	A	4
10	Isoleucine	Ile	I	3
11	Isoleucine	Ile	I	3
12	Proline	Pro	P	4
13	Arginine	Arg	R	6
14	Glutamine/ Glutamic acid	Glx	Z	2/2
15	Aspartic acid	Asp	D	2
16	Threonine	Thr	T	4
17	Leucine	Leu	L	6
18	Threonine	Thr	T	4
19	?			
20	Proline	Pro	P	4
21	?			
22	?			
23	Asparagine	Asn	N	2
24	Serine	Ser	S	6



**Figure 5.5.** Comparison of the TaHBL20/125 antigen and human ICAM-1 amino acid sequences.

**Figure 5.6.** Comparison of the N-terminal amino acid sequence of human ICAM-1 with those of mouse, rat, dog and with the amino acid sequence of the TaHBL20/125 antigen. Dashed lines represent amino acids identical to the human sequence. The N-terminal residue of the first Ig like extracellular domain is marked with an arrow. Residues discussed in the text as important for human ICAM-1 binding to LFA-1 are boxed.

TaHBL20/1253S I A P S K A I I P R13

| : . | | | | : | |

ICAM-130S V S P S K V I L P R40

- | Exact match
- : Closely related
- . Related

Percentage similarity82%

Percentage identity64%

150

HumanMAPSSRPALPALLVLLGALFPGPGNAQTSVSPSKVILFRGGSVLVTCST

Mouse--STRAK-T--L--A-VTVVI----D--V-IH-REAFL-Q----Q-N--S

Rat--STRA--M--L----VAVVI---VG--V-IH-TEAFL-- ----Q-N--S

Dog--SAPAL-R- ----A-----L--L-G---- -D-AEA-I- ----Q-N---

TaHBL20-IA---A-I--ZDTLT

/125L>

**Table 5.3.** Comparison of the percentage similarity and percentage identity between the human ICAM-1 amino acid sequence and those of mouse, rat and dog over the entire length of the molecule, and over the region of homology corresponding to the TaHBL20/125 amino acid (aa) sequence (residue 30 to 40 in human ICAM-1, see Figure 5.5.) .

	Entire molecule		TaHBL20/125 aa region	
	% similarity	% identity	% similarity	% identity
Rat ICAM-1	58	52	54	45
Mouse ICAM-1	58	42	45	36
Dog ICAM-1	56	52	54	45
TaHBL20/125	---	---	82	64

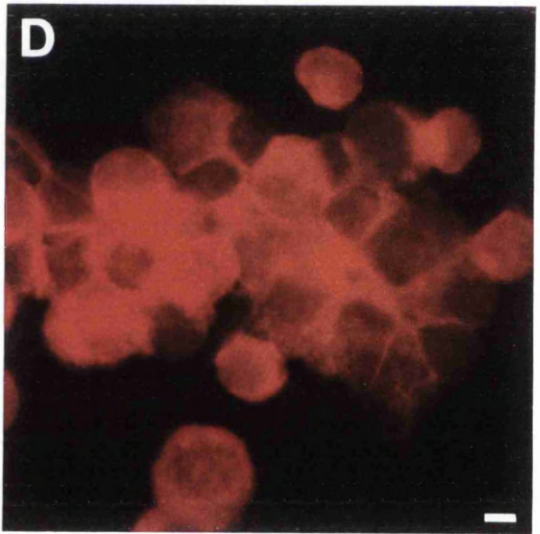
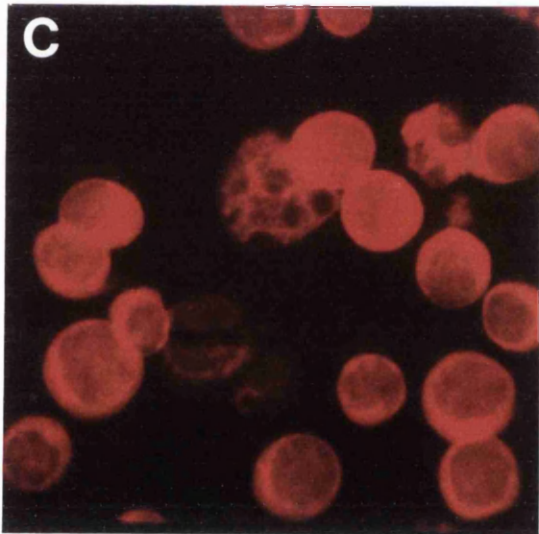
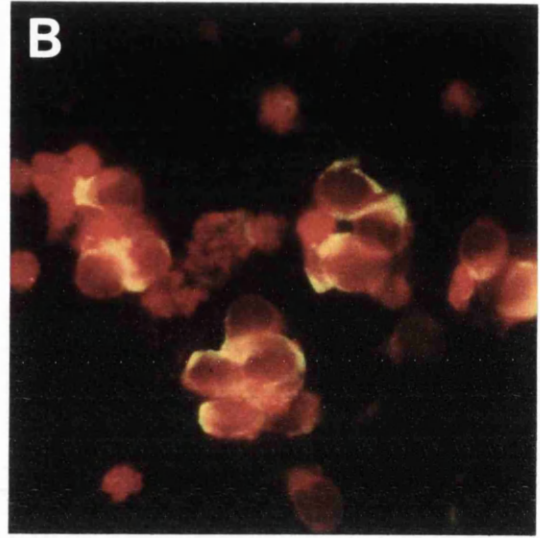
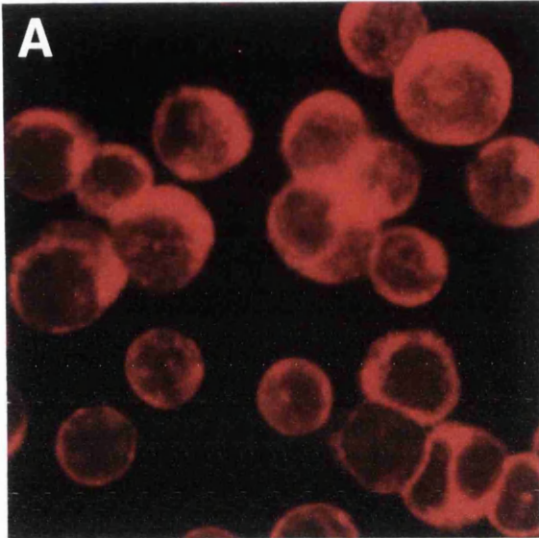
obtained when the comparison was made over the region corresponding to the TaHBL20/125 amino acid sequence. In contrast, TaHBL20/125 showed a 64% identity with human ICAM-1 over this region, indicating that the homology of TaHBL20/125 was higher than that observed with any other species.

To further investigate the relationship between TaHBL20/125 and human ICAM-1, fixed slide preparations of infected TaHBL20 and uninfected BL20 cells were tested for reactivity by IFA with an anti-human ICAM-1 monoclonal antibody. As a control HL-60 cells were also tested for reactivity. Normal HL-60 cells showed no reactivity with the anti-ICAM-1 monoclonal antibody (Figure 5.7.A). Following induction of differentiation towards macrophages with PMA (see 3.2.1.) most of the cells displayed bright positive reactivity (Figure 5.7.B). No reactivity was observed using this antibody with either BL20 or TaHBL20 (Figure 5.7. C and D).

### **5.3.3. Oligonucleotide screening of a $\lambda$ Zap D7/37 cDNA library**

The amino acid sequence data obtained for TaHBL20/125 was used to design oligonucleotide pools to screen the  $\lambda$  Zap D7/37 cDNA library. Due to the redundancy of the genetic code, the nucleotide coding sequence of TaBL20/125 contained codons with twofold, threefold, fourfold and sixfold ambiguity (Table 5.2.). This made the design of oligonucleotides difficult. To synthesis the oligonucleotide pools, nucleotide sequence derived from the amino acid sequence starting at residue 8 (lysine) to residue 13 (arginine) was chosen, as this represented the longest and least degenerate stretch of amino acids. A comprehensive pool of oligonucleotides representing all possible nucleotide coding sequences for the peptide was calculated as having 576 possible nucleotide coding sequences ( $2 \times 4 \times 3 \times 3 \times 4 \times 2$ ). To lower this degeneracy, inosine was included to replace the first nucleotide of

**Figure 5.7.** Immunofluorescence reactivity of an anti-human ICAM-1 monoclonal antibody on fixed slide preparations of; (A) HL-60 cells; (B) HL-60 cells induced to differentiate towards macrophages by a 24 hour incubation with 20 ng ml<sup>-1</sup> PMA; (C) BL20 cells; (D) TaHBL20 cells. (Bar = 10 µm).



the codon for arginine at position 13, and the oligonucleotide was truncated at the second base of this codon. These modifications reduced the number of coding sequences required to 288 ( $2 \times 4 \times 3 \times 3 \times 4$ ). A further reduction in degeneracy was accomplished by synthesis of the oligonucleotides in four separate pools, each containing 72-fold degenerate, 17-base oligonucleotides (Figure 5.8.). This was predicted to increase the molar concentration of the exact match oligonucleotide sequence within the pool, which would enhance the probability of detection of a positive plaque.

As the pools of oligonucleotides comprised of individual sequences with variable G and C contents, it was impossible to estimate the consensus melting temperature of hybridisation ( $T_m$ ) for the pool as a whole. However, using the formula given by Itakura, *et al.* (1984) it was possible to calculate the lowest and highest possible  $T_m$  for each oligonucleotide pool to be 44°C and 54°C respectively (Figure 5.8.). In order to maximise the number of positive clones isolated, the *T. annulata* D7/37 cDNA library in  $\lambda$  Zap was screened using conditions of very low stringency (see 5.2.6.). All hybridisations were carried out at 37°C, *i.e.* 7°C below the lowest calculated  $T_m$ , and the filters were washed at 37°C with 6 x SSC, 0.5 % SDS. A total of  $1.2 \times 10^5$  recombinant phage were screened with each of the 4 oligonucleotide pools. 5 positive plaques were identified and purified to homogeneity by successive rounds of screening (Figure 5.9.).

#### **5.3.4. Characterisation of the $\lambda$ Zap clones**

To aid in the isolation of DNA from the  $\lambda$  Zap clones phagemids of each clone were isolated by *in vivo* excision into the pBluescript SK (+/-) phagemid vector (see 5.2.7.). The phage clones were allowed to infect cells which were co-infected with the filamentous helper phage R480. Inside the cell, trans-acting proteins from the helper phage recognise two separate

**Figure 5.8.** Design of oligonucleotide pools (POA1 to 4) taking into account the variability in nucleotide triplets which encode the amino acid sequence of the TaHBL20/125 antigen. Melting temperature of hybridisation ( $T_m$ ) for the oligonucleotides was estimated according to (Itakura, *et al.*, 1984). For the lowest  $T_m$ , the variant nucleotide is assumed to be either A or T. For the highest  $T_m$  the variant nucleotide is assumed to be either G or C.



Nº	8			9			10			11			12			13		
aa	lys			ala			ile			ile			pro			arg		
seq	A	A	A	G	C	T	A	T	T	A	T	T	C	C	T	A	G	
			G			C			C			C			C	C		
						A			A			A			A			
						G									G			

<u>POA1</u>	A	A	R	G	C	Y	A	T	H	A	T	H	C	C	Y	I	G	
<u>POA2</u>	A	A	R	G	C	Y	A	T	H	A	T	H	C	C	R	I	G	
<u>POA3</u>	A	A	R	G	C	R	A	T	H	A	T	H	C	C	Y	I	G	
<u>POA4</u>	A	A	R	G	C	R	A	T	H	A	T	H	C	C	R	I	G	

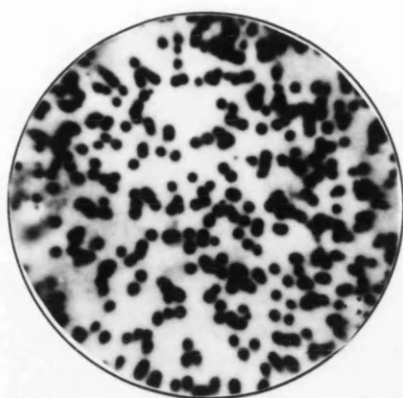
Y= C or T                    R= A or G  
H= T, C or A                I= inosine

Tm: 2(A+T+I) + 4(G+C)

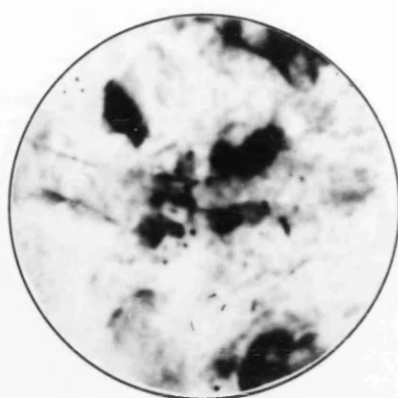
Lowest Tm: 2(12) + 4(5) = 44°C  
Highest Tm: 2(7) + 4(10) = 54°C

**Figure 5.9.** Plaque lifts of  $\lambda$  Zap D7/37 cDNA clones following tertiary screening using degenerate oligonucleotides POA1, 2 and 4.

Letter	Clone	Oligonucleotide
A	1C1A	POA1
B	1E1A	POA1
C	negative control	POA1
D	2C1A	POA2
E	negative control	POA2
F	4A1	POA4
G	4E1A	POA4
H	negative control	POA4



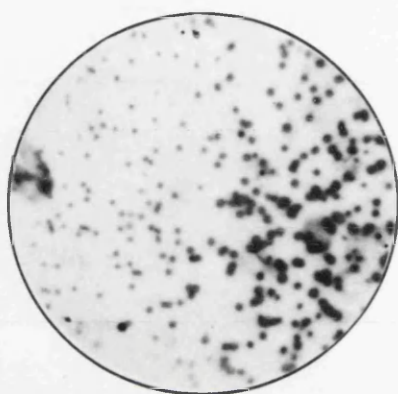
A



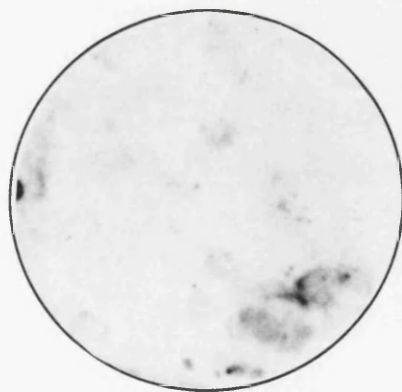
B



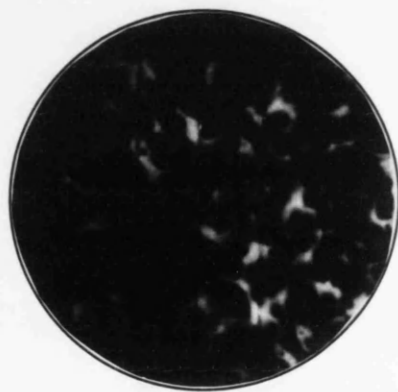
C



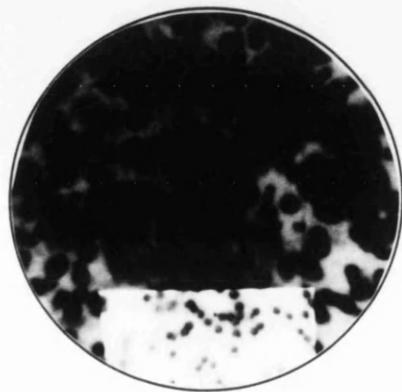
D



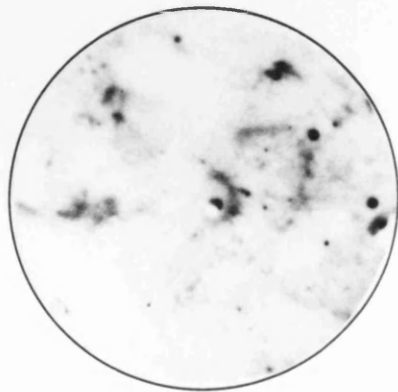
E



F



G



H

domains (initiator and terminator) positioned within the  $\lambda$  Zap vector arms. Both of these signals are recognised by the helper phage gene II protein and a new DNA strand is synthesised. This strand is circularised and packaged as a filamentous phage by the helper phage proteins and secreted from the cell. The resulting pBluescript plasmids were then recovered by infecting the F' containing XL1-Blue MRF' bacterial strain, and plasmid DNA was prepared as described in 5.2.8. To estimate the size of the  $\lambda$  Zap cloned inserts, DNA was restriction digested with *Sma*I and *Kpn*I or *Pst* I and *Apa* I (see 5.2.10.), and the products analysed by agarose gel electrophoresis (Figure 5.10.). The pBluescript SK (+/-) vector DNA is visible as a band of approximately 3 kb (Figure 5.10 lanes 1 to 5). Analysis of the digested DNA from the clones showed additional DNA fragments corresponding to the inserted cDNA. Clones 1C1A, 1E1A, 2C1A and 4E1A had insert sizes in excess of 3.5 kb, while 4A1 had the smallest insert of only 1.6 kb (Table 5.4.).

The 5' and 3' end of each insert was then sequenced using the T3 and T7 primers respectively (see 5.2.14.). The  $\lambda$  Zap D7/37 cDNA library was constructed such that, the 5' of each cDNA clone was inserted into the *Eco*RI site of the  $\lambda$  Zap vector using *Eco*RI adapters and the 3' of each cDNA was inserted into the *Xho* I site of the vector using *Xho* I adapters. Therefore, the 5' end of each cDNA clone should have corresponded to the N-terminal amino acid sequence of the peptide encoded by that sequence. The orientation of the cloned cDNA inserts was confirmed as tracts of poly A sequence were seen at the 3' end of each cDNA clone. Comparison of the nucleotide sequence or predicted 5' peptide sequences of each clone to the TaHBL20/125 N-terminal peptide or the predicted nucleotide sequence failed to identify any homology (see appendix). Thus, it was concluded that none of the isolated cDNA clones encoded the polypeptide of interest.

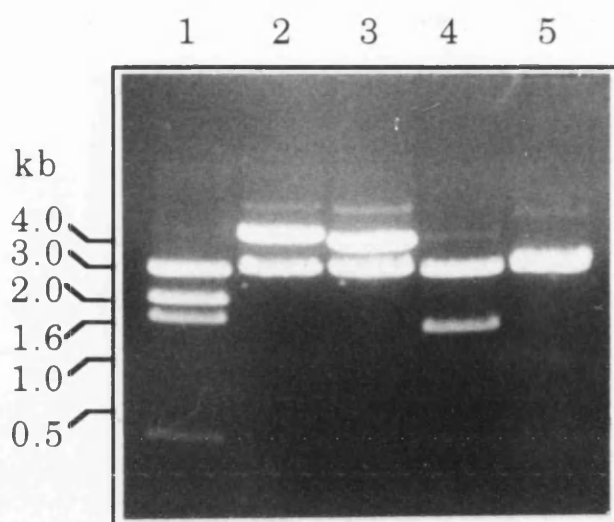
**Figure 5.10.** Agarose electrophoresis analysis of c DNA clones isolated from the  $\lambda$  Zap D7/37 cDNA library. cDNA clones were digested with restriction enzymes; (A) *Kpn* I and *Sma* I or (B) *Pst* I and *Apa* I to release insert DNA.

Lane 1 1C1A  
 Lane 2 1E1A  
 Lane 3 2C1A  
 Lane 4 4A1  
 Lane 5 4E1A

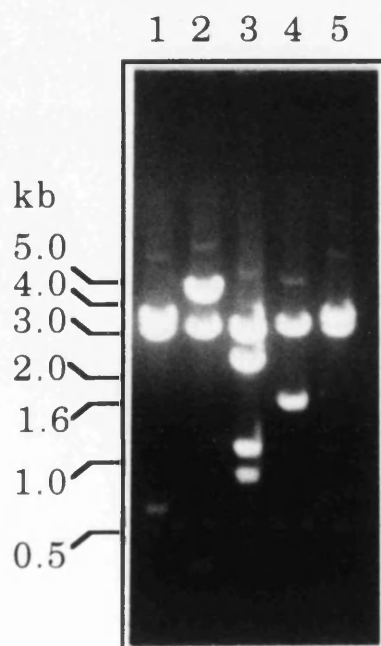
**Table 5.4.** Approximate size of the inserts of cDNA clones estimated by restriction digest and agarose gel electrophoresis.

Clone	Size of insert (kb)
1C1A	4.3
1E1A	5.5
2C1A	4.5
4A1	1.6
4E1A	3.5

(A)



(B)



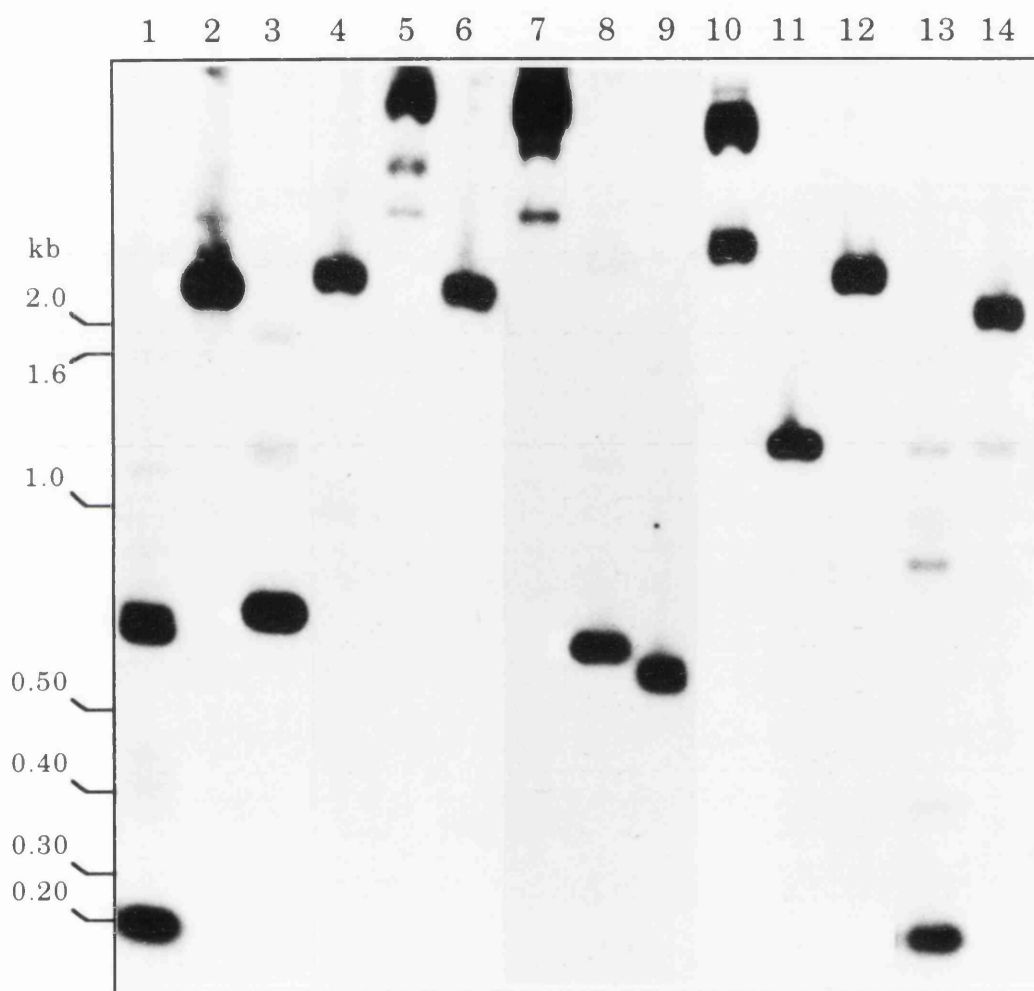
It was possible that the sequence encoding TaHBL20/125 might not be present at the 5' end of the cDNA clone. Therefore, rather than sequencing the entire DNA insert of each clone, smaller DNA fragments, which hybridised to the appropriate oligonucleotide probe, were identified and subcloned. DNA from each clone was digested with a number of restriction enzymes and the products analysed by agarose gel electrophoresis. The gel was then Southern blotted (see 5.2.11.) and hybridised with the appropriate oligonucleotide probe. Smaller DNA restriction fragments were identified which were detected by the pooled oligonucleotide probes (Figure 5.11.). Fragments generated by digestion with the same restriction enzymes were then purified by low melting point agarose gel electrophoresis and subcloned into the pBluescript SK (+/-) vector (see 5.2.12.). This procedure was carried out for all the original positive clones except clone 1E1A. When DNA from this clone was digested with restriction enzymes, it did not produce any small fragments which hybridised with the POA1 oligonucleotide probe (Figure 5.11. lanes 4 to 6), and time constraints did not permit any further investigation of this clone.

DNA was prepared from six white colonies of each of the subclones, DNA inserts were released by digestion with restriction enzymes, and analysed by agarose gel electrophoresis. Positive subclones were identified by Southern blotting with the appropriate oligonucleotide probe (Figure 5.12). 1C1A subclones 1, 3 and 5 contained hybridising DNA fragments of the expected size (0.7 kb) and 3 was selected for subsequent sequence analysis (Figure 5.12.A). Subclones 2 and 4 of 2C1A contained hybridising DNA fragments of the expected size (0.6 kb) and 2 was subsequently sequenced (Figure 5.12.B.). All 6 subclones of 4A1 contained hybridising DNA fragments of the expected size (1.2 kb) and 2 was subsequently sequenced

**Figure 5.11.** Southern blot analysis of cDNA clones following digestion with a variety of restriction enzymes and probing with degenerate oligonucleotides as indicated.

Lane	Clone	Restriction enzyme	Probe	Size of subcloned fragment
1	1C1A	<i>Sau</i> 3A I	POA1	0.7 kb
2	1C1A	<i>Msp</i> I	POA1	
3	1C1A	<i>Rsa</i> I	POA1	
4	1E1A	<i>Mbo</i> I	POA1	
5	1E1A	<i>Msp</i> I	POA1	0.6 kb
6	1E1A	<i>Rsa</i> I	POA1	
7	2C1A	<i>Mbo</i> I	POA2	
8	2C1A	<i>Msp</i> I	POA2	1.2 kb
9	2C1A	<i>Rsa</i> I	POA2	
10	4A1	<i>Mbo</i> I	POA4	
11	4A1	<i>Msp</i> I	POA4	0.25 kb
12	4A1	<i>Rsa</i> I	POA4	
13	4E1A	<i>Sau</i> 3A	POA4	
14	4E1A	<i>Msp</i> I	POA4	





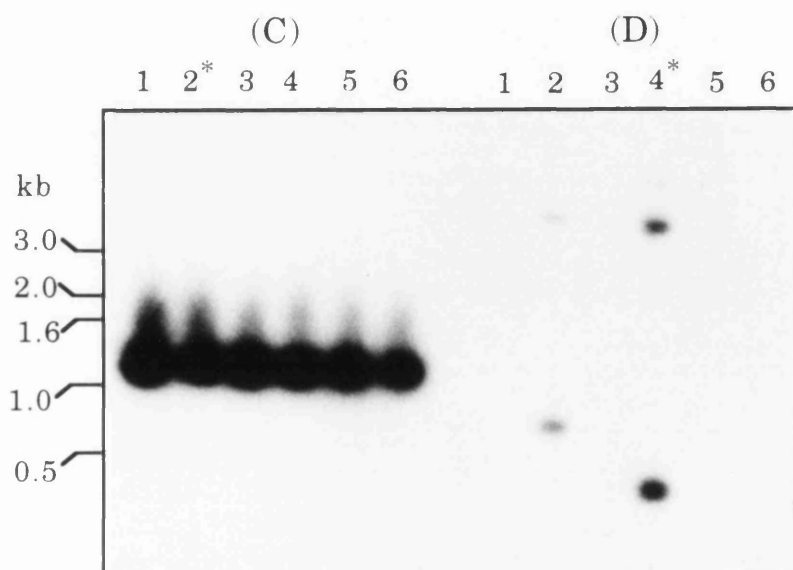
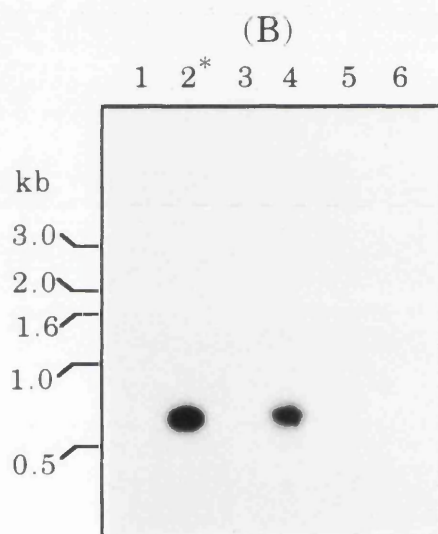
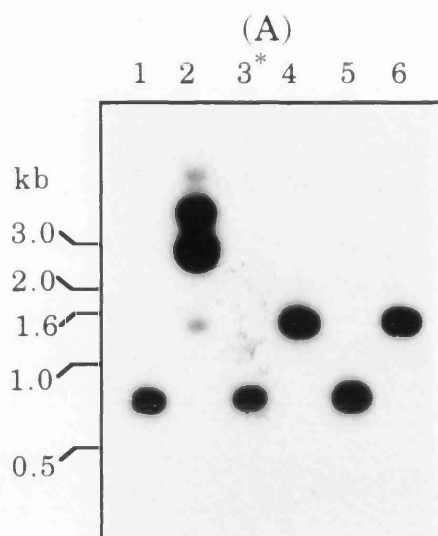
**Figure 5.12.** Southern blot analysis of cDNA subclones following digestion with *Bam*HI and *Eco*RI restriction enzymes and probing with degenerate oligonucleotides as indicated. A \* indicates the subclone subsequently selected for sequencing.

A; 1C1A subclones 1-6 probed with oligonucleotide POA1

B; 2C1A subclones 1-6 probed with oligonucleotide POA2

C; 4A1 subclones 1-6 probed with oligonucleotide POA4

D; 4E1A subclones 1-6 probed with oligonucleotide POA4



(Figure 5.12.C). 4E1A subclone 4 contained a hybridising DNA fragment of the expected size (0.25 kb) and a higher band (3.5 kb) which was thought, from the size estimation, to represent a partial digestion product rather than a repetition of the oligonucleotide target sequence (Figure 5.12.D). The smaller DNA fragments of the four subclones were sequenced in both directions. Comparison of the nucleotide sequence or deduced peptide sequences of each clone to that of the N-terminal sequence obtained for TaHBL20/125 or the deduced nucleotide sequence failed to identify any of the subcloned inserts as having homology with the antigen recognised by monoclonal antibody 4H5. It was concluded therefore, that the gene encoding TaHBL20/125 had not been identified using the approach described above.

## 5.4. Discussion

To clone the gene encoding TaHBL20/125, immunoaffinity purification of the antigen, followed by peptide sequencing was performed. To maximise the amount of antigen obtained, TaHBL20 extracts were first enriched for glycoproteins by Con A lectin affinity chromatography before passage over a 4H5 immunoaffinity column. Comparison of immunoblots of eluates following passage over the lectin column (Figure 5.2.) with eluates following passage over the monoclonal antibody 4H5 biotin/streptavidin column (Figure 5.3.) showed that complete purification of TaHBL20/125 had been achieved. In addition, when a sample of the purified antigen was run on an acrylamide gel and stained with Coomassie blue no other contaminating polypeptides, which may not have been recognised by the anti-glycoprotein antibody, were present (Figure 5.4.).

An N-terminal peptide sequence of 18 amino acids was obtained and when this sequence was compared to other polypeptides in the Swiss Prot data base by BLAST search, a 82% similarity, 64% identity to human ICAM-1 was found. ICAM-1 is a member of the immunoglobulin superfamily (Staunton, *et al.*, 1988) and is a single chain heavily glycosylated trans-membrane protein, which has five extracellular Ig-like domains. The molecule displays a variable molecular weight of between 76 kDa and 114 kDa dependant on tissue specific glycosylation (Clark, *et al.*, 1986; Dustin, *et al.*, 1986). Cell adhesion is mediated by the binding of ICAM-1 to the cell surface ligand LFA-1 (lymphocyte function-associated antigen-1) which is expressed on all leukocytes (Dustin, *et al.*, 1986; Rothlein, *et al.*, 1986). Interaction of ICAM-1 and LFA-1 is essential for cell mediated cytotoxicity (Schmidt, *et al.*, 1985; Shaw, *et al.*, 1986), the interaction of T and B cells (Sanders, *et al.*, 1986) and the homotypic adhesion of monocytes and B cells

(Mentzer, *et al.*, 1986; Mentzer, *et al.*, 1985). In addition ICAM-1 is necessary for lymphocyte-endothelial cell adhesion and facilitates lymphocyte migration to the sites of inflammation (Dustin and Springer, 1988).

Comparison of the human ICAM-1 amino acid sequence to the ICAM-1 sequences of different species indicates a 42% to 52% identity over the entire molecule (Table 5.3.). When a similar comparison is made over the region corresponding to the TaHBL20/125 peptide sequence, the percentage identity of different species to human ICAM-1 is much lower. In contrast, TaHBL20/125 shows a 64% identity with human ICAM-1. A similar high percentage of homology was recently reported for the bovine ICAM-3, where this molecule was found to have 61% and 58% identity to human ICAM-3 and ICAM-1 respectively (Lee, *et al.*, 1996). This suggests that ICAM-1 polypeptides are more closely related between bovines and humans compared to other species analysed

A comparison of the N-terminal ICAM-1 amino acid sequences from various species and the obtained amino acid sequence of TaHBL20/125 was made (Figure 5.6.). The obtained amino acid sequence of TaHBL20/125 was found to be homologous to the start of the first Ig like domain of ICAM-1 (Staunton, *et al.*, 1990). The preceding amino acids correspond to the signal peptide which ensures surface expression of ICAM-1 (Manning, *et al.*, 1995; Staunton, *et al.*, 1990). TaHBL20/125 is known to be located on the surface of the infected cell (Shiels, *et al.*, 1989 and Chapter 2), therefore, it is likely that the corresponding signal peptide on the TaHBL20/125 would have been removed during transport of the antigen to the cell surface. The arginine<sup>40</sup> and glycine<sup>41</sup> (Figure 5.6.) residues in the N-terminus of ICAM-1 have been established as important for the binding to LFA-1 (Staunton, *et al.*, 1990)

and are conserved in all but murine ICAM-1. Unfortunately, although TaHBL20/ was found to contain an arginine at residue 13, it did not contain a glycine at residue 14 (Table 5.2.). Clearly, the presence of both these residues would support the identification of TaHBL20/125 as the bovine homologue of ICAM-1. A monoclonal antibody against human ICAM-1 failed to show any reactivity with *Theileria* infected cells (Figure 5.7. D.). This in itself does not preclude the possibility that TaHBL20/125 is the bovine homologue of ICAM-1, as the specific epitope for the antibody could be absent in the bovine molecule. It is also unknown whether this monoclonal antibody cross reacts with the bovine homologue. Alternatively, it is possible that TaHBL20/125 represents a related CAM with structural motifs which are conserved in human ICAM-1.

There are striking similarities in the general size, location and variation in molecular mass observed between ICAM-1 and TaHBL20/125. *In vitro*, cells infected by *T. annulata* are characteristically adherent to each other, often forming large clumps in culture media which are clearly visible. It would seem likely therefore, that host cell adhesion molecules are up-regulated following infection by the parasite. Studies on *T. parva* have shown that continuous proliferation requires surface stimulation mediated through cell-cell contact (Dobbelaere, *et al.*, 1991). The ability to respond to surface stimulation was found to be dependant on the continued presence of the parasite, as removal by theilericidal drugs resulted in the loss of the response. These experiments demonstrate a role for cell-cell contact in *Theileria* infected cells and implicate molecules such as ICAM-1 in this important process.

ICAM-1 is only normally detected in very few cell types (Rothlein, *et al.*, 1986). However, expression of ICAM-1 is rapidly up-regulated *in vitro* and *in vivo* by proinflammatory cytokines such as IFN- $\gamma$ , IL-1 $\beta$  and TNF- $\alpha$  (Dustin, *et al.*, 1986; Lane, *et al.*, 1989; Springer, *et al.*, 1987). These cytokines have been shown to be produced by *Theileria* infected cells (Ahmed, *et al.*, 1993; Brown, *et al.*, 1995; Preston, *et al.*, 1993) and could therefore be implicated in the mechanism of ICAM-1 up-regulation in infected cells. In addition, the transcriptional regulation of ICAM-1 is known to be controlled, at least in part, by the transcription factor NF- $\kappa$ B (Voraberger, *et al.*, 1991). Increased expression of this transcription factor has also been shown in *Theileria* infected cells (see 1.7.), allowing a putative connection to be made between the changes in gene expression known to accompany immortalisation by the parasite and the possible expression of ICAM-1.

Peptide sequence data had suggested that TaBL20/125 could be a CAM, but in order to corroborate this finding it was necessary to clone the representative cDNA and obtain nucleotide sequence data. By back-translating the amino acid sequence it was hoped that oligonucleotides could be designed which would allow the screening of a cDNA library to be undertaken. Unfortunately, the amino acid sequence data obtained was highly redundant which made the design of oligonucleotide probes problematic. A possible way of overcoming this redundancy would be to obtain additional internal sequence by generating peptide fragments by limited proteolysis of the remaining purified antigen. However, although this was attempted, the peptide fragments sequenced were found to be autolytic digestion products of the protease enzymes used (data not shown).



Two possible approaches are available for the design of oligonucleotide probes from degenerate amino acid sequence. Either a guessmer can be designed based on the codon bias displayed by bovine genes or oligonucleotide pools containing all possible coding sequences can be synthesised. When designing a guessmer an oligonucleotide probe could be expected to have at least 76% homology with the target gene by chance, and this can be increased to over 80% if the codon utilisation of the species of interest is known (Sambrook, *et al.*, 1989). Comparison of the sequences of guessmers with those of the target gene show that successful identification can be achieved with homologies as low as 71% (Derynck, *et al.*, 1985). However, almost all guessmer oligonucleotide probes which have been used successfully contain regions that match exactly with the target sequence (Sambrook, *et al.*, 1989). Codon bias is both species and tissue specific and at the time when the oligonucleotides were designed, few bovine CAMs had been identified. In addition, as it was not possible to incorporate an exactly matching stretch of sequence into the guessmer, it seemed that using a pooled oligonucleotide approach had a greater chance of being successful.

Degenerate oligonucleotide probes have been used successfully to clone target genes. For example, Orkin, *et al.* (1983) used a 17mer with 64 fold degeneracy to clone the human deaminase gene and Bell, *et al.* (1984) used a 23mer with 256 fold degeneracy to clone the gene for human preproinsulin-like growth factor. The longest and least degenerate stretch of amino acid sequence for TaHBL20/125 antigen was used to design 4 oligonucleotide pools each containing 72 of the 288 possible coding sequences (Figure 5.8.). A further complication of using degenerate oligonucleotide probes is encountered when trying to optimise hybridisation conditions. As the target sequence was unknown, the  $T_m$  of the matching oligonucleotide could not be accurately estimated. Conditions had to be

used which allowed the oligonucleotide with the lowest G + C content to hybridise efficiently. One possible way to overcome this problem is to hybridise the probes in solvents which contain tetramethylammonium chloride (TMACl). In these solvents, the  $T_m$  of a hybrid is independent of its base composition and dependant primarily on its length (Jacobs, *et al.*, 1985; Wood, *et al.*, 1985). However, preliminary experiments using this method were unsuccessful. These screenings gave an unacceptable amount of background hybridisation, making the identification of positive clones impossible. In view of this, subsequent hybridisations were carried out in PIPES buffer at a lower temperature of 37°C, *i.e.* 7°C below the lowest possible calculated  $T_m$  (see 5.2.6.). Using such low stringency conditions can lead to the isolation of high numbers of false positives, as mismatched hybrids formed with oligonucleotides of a higher G + C content may be more stable than the perfectly matching hybrid.

DNA was isolated from the five positive clones detected by the screening procedures and the size of the inserts estimated. The antigen in TaHBL20 was 125 kDa, therefore it was possible to estimate that the DNA coding sequence for this molecule should be approximately 2.0 kb (100 kDa = 1.575 kb, (Sambrook, *et al.*, 1989)). However, because the molecule was known to be glycosylated and the contribution made by post-transcriptional modifications to the overall size of the polypeptide was unknown, the DNA coding sequence for this molecule could be smaller than estimated. Therefore, while all the clones apart from 4A1 had insert sizes of 2.0 kb or larger, the 4A1 clone with an insert size of 1.6 kb could not be discounted on size alone.

The orientation of the cDNA inserts was confirmed by sequencing of the 3' ends. The N-terminal of the polypeptide corresponded to the 5' terminal of

the DNA sequence. Therefore, if the 5' end of the cloned DNA sequences were translated, the TaHBL20/125 amino acid sequence should be present. At least 0.4 kb of the 5' end of each cDNA clone was sequenced but no homology was found between the predicted coding sequences of the cDNA clones and the peptide sequence of TaHBL20/125. In addition, comparison of clone sequences with nucleotide and amino acid sequences in the data base did not identify any of the clones as having homology with known genes. To ensure that the region to which the oligonucleotides were binding had been sequenced, DNA from the cDNA clones was digested with a range of restriction enzymes and Southern blotted with the relevant oligonucleotide probe. Small DNA restriction fragments, which bound the oligonucleotides were identified and sub-cloned. The inserts were sequenced in both directions, however no homology to the 4H5 amino acid sequence or deduced nucleotide sequence was found in any of the subclones.

Although disappointing, it is not entirely surprising that the library screening failed to identify the gene encoding the antigen recognised by monoclonal antibody 4H5. The amino acid sequence obtained for this antigen was so degenerate that it made the design of specific oligonucleotide probes extremely difficult. It is possible that some of the oligonucleotides within each pool would bind to DNA sequences in the library by chance, leading to the isolation of false positives. Although one of the oligonucleotide pools should have contained an exact match to the target gene, optimising the hybridisation conditions for this sequence was not possible. In addition, the low stringency hybridisation conditions which were used would also increase the probability of isolating false positives. It seemed likely therefore, that the inherent limitations in the cloning strategy led to the non-specific hybridisation and random isolation of the cDNA clones. In the absence of further internal sequence to confirm the

isolation of cDNA clones, this strategy always contained appreciable risk of failure. Clearly any future studies would either have to obtain further amino acid sequence data, or use an alternative approach such as the design of guessmers base on the recently published bovine ICAM-3 sequence (Lee, *et al.*, 1996) or oligonucleotide probes based on CAM consensus nucleotide sequences, to clone the gene encoding TaHBL20/125.

## **Chapter 6**

## General Discussion

One of the primary aims of the work presented in this thesis was to identify the gene encoding the variable molecular weight antigen recognised by monoclonal antibody 4H5 in *T.annulata* infected cells. This molecule was thought to represent a host molecule up-regulated by the presence of the parasite within the host cell. Identification of the gene encoding TaHBL20/125 would, therefore, provide a model system for further investigations into the mechanism of the parasite control of host cell gene expression.

Attempts to establish a direct association between infection by the parasite and the induction of TaHBL20/125 expression were inconclusive. Infection of the lymphosarcoma cell line BL20 with *T. annulata* sporozoites did not immediately induce expression of TaHBL20/125, but expression was observed after the newly infected cell lines were subjected to prolonged culture *in vitro*. These findings were in contrast to the results of a similar study (Leila Ben-Miled, unpublished data) in which peripheral blood leukocytes were shown to express TaHBL20/125 soon after infection by the parasite. It was concluded that sporozoite infection of PBLs was probably more indicative of the *in vivo* situation, as it was unknown how infection of an already transformed cell line might influence the normal sequence of immortalisation events brought about by the parasite. Nevertheless, alterations to the BL20 cell line caused by the presence of the parasite had clearly taken place, as soon after infection large clumps of infected cells were seen in the cultures and the rate of cell proliferation was increased. In the future, it may be of interest to examine the process of altered gene expression in the transformed BL20 cell line compared to normal host cells. Such studies could determine which alterations are a primary result of

infection and, whether the parasite alters the control of host cell proliferation in a manner distinct from other mechanisms or at a different point in a common immortalisation pathway.

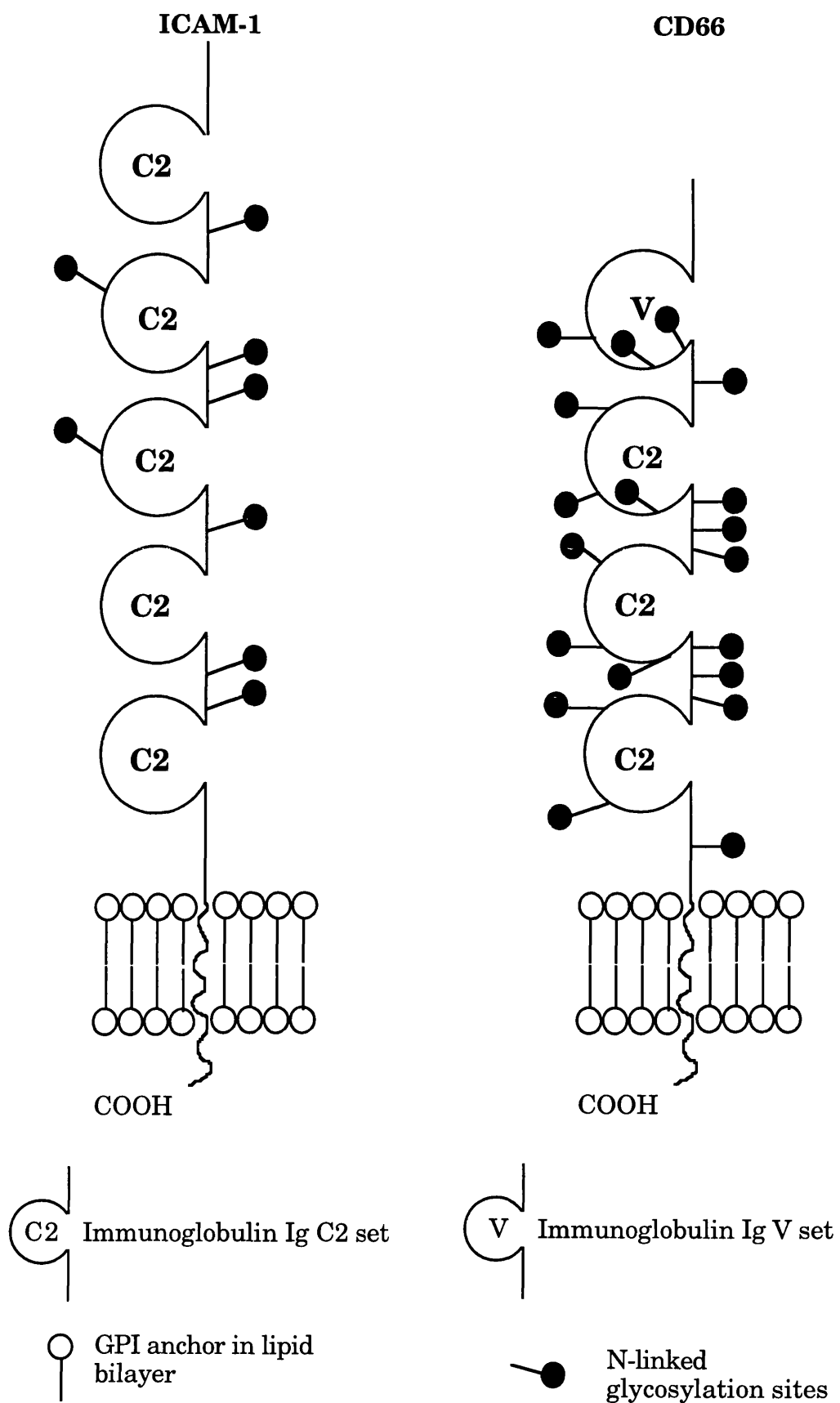
As TaHBL20/125 was assumed to be host derived, it might be expected to be expressed on cells of a related lineage. When the human leukemic cell line HL-60 was examined it was found that monoclonal antibody 4H5 recognised a molecule of 160 kDa on the surface of these cells. Furthermore, expression of this antigen was found to increase, in a lineage specific manner, following differentiation towards granulocytes. Recognition of TaHBL20/125 by monoclonal antibody 4H5 was found to be sensitive to conditions known to alter the structural integrity of polypeptides. This was also found to be the case in HL-60 cells, where recognition of the g160 kDa antigen by the antibody on immunoblots was entirely dependant on the use of non-denaturing conditions and treatment with periodate.

Sufficient amounts of TaHBL20/125 were purified by immunoaffinity chromatography to allow peptide sequencing to be performed. The N-terminus of the antigen was found to have significant homology to human ICAM-1. This was of particular interest, as infection by the parasite is known to induce alterations to the surface of the host cell which enable infected cells to adhere to each other or to the surface of the culture vessel. In addition, cell-cell contact has been shown to be important in the proliferation of *T. parva* (Dobbelaere, *et al.*, 1991). Some of the similarities between ICAM-1 and TaHBL20/125 could be extended to the g160 kDa antigen in HL-60, though there are two reasons why g160 may not represent ICAM-1. Firstly, ICAM-1 has a variable molecular weight of between 76 kDa and 114 kDa and the antigen detected by monoclonal antibody 4H5 in HL-60 granulocytes was found to be 160 kDa. Secondly,

expression of ICAM-1 in HL-60 is induced following differentiation towards macrophages and granulocytes (Staunton, *et al.*, 1988; Triozzi, *et al.*, 1992), whereas expression of the g160 antigen was found to be restricted to granulocytes only. However, the difference in size between TaHBL20/125 and g160 could be accounted for by different post-transcriptional or post-translational modifications to homologues, as these types of modification have been well documented for CAMs, including ICAM-1 (Clark, *et al.*, 1986; Dustin, *et al.*, 1986; Reyes, *et al.*, 1991). It is also possible that post-translational or post-transcriptional differences could result in the loss of the epitope for monoclonal antibody 4H5 in macrophages, resulting in the inability of the antibody to recognise g160 in this cell type.

Striking similarities were found between g160 and the CD66 homotypic cell adhesion molecule. Both these molecules are the same size and are induced in HL-60 following differentiation towards granulocytes. A comparison of the structural domains found in ICAM-1 and CD66 shows that both molecules contain immunoglobulin domains of the C2 set (Figure 6.1.). It is possible therefore, that the antigens recognised by monoclonal antibody 4H5 in *T. annulata* infected cells and in HL-60 granulocytes, represent closely related molecules which could share structural epitopes for the antibody. CD66 is a member of the CEA subgroup and cross reactivity of monoclonal antibodies for shared epitopes has been documented as a major problem for the assignment of members to this group (Engvall, *et al.*, 1978; Paxton, *et al.*, 1987; Shively and Beatty, 1985; Thompson, *et al.*, 1987). Thus, there are a number of candidate molecules which could be the homologue of the infection associated antigen recognised by monoclonal antibody 4H5. Definitive identification could only be made by obtaining further data, which ideally would be at the amino acid level.





**Figure 6.1.** Model of domains found in leukocyte membrane proteins ICAM-1 and CD66. Adapted from Barclay, A. N. *et al* (1993)

To identify the gene encoding the TaHBL20/125 antigen screening of a cDNA library was undertaken. Pooled oligonucleotide probes were designed from the amino acid sequence data obtained for TaHBL20/125. Considerable difficulties were encountered in both the design of the oligonucleotides and in the establishment of specific hybridisation conditions. Unfortunately, no clones encoding the TaHBL20/125 gene were isolated. The sensitivity of monoclonal antibody 4H5 to factors affecting the structural integrity of its epitope was a major limitation when considering the various methods available with which to clone the gene. One way of possibly overcoming these difficulties would be to utilise the protocols established here to purify greater amounts of TaHBL20/125. Purified antigen could then be used to raise antisera, which might specifically recognise denatured TaHBL20/125 antigen. The isolation of a less sensitive, specific antibody would be predicted to increase the chances of isolating the gene encoding TaHBL20/125 using immuno-screening of *Theileria* infected cell or mammalian cDNA expression libraries. In addition, purification of larger amounts of antigen might also enable further experiments to be undertaken using proteolytic cleavage to generate smaller and, possibly, less degenerate fragments of the antigen for peptide sequencing.

It would be of interest to examine if host CAMs are up-regulated directly by the parasite, and perhaps perform a specific function during infection. Alternatively expression of CAMs may play no role in the course of infection and could be the result of a secondary event brought about by increasing levels of transcription factor NF- $\kappa$ B, as this alteration has been associated with the parasite induced transformation (Ivanov, *et al.*, 1989) and is known to be directly involved in the control of ICAM-1 expression (Voraberger, *et al.*, 1991).

Other studies have also characterised phenotypic changes resulting from infection associated immortalisation and modification of host cell gene expression (see 1.7.). These alterations, however, could be secondary events linked to transformation, which are influenced by the developmental status of the infected cell. The challenge for current and future research is to define the primary point of interaction between parasite derived factors and host cell regulatory proteins which bring about immortalisation and essential changes to host cell gene expression.

Characterisation of the g160 kDa antigen in HL-60 cells, and the finding that expression of this antigen was increased following differentiation towards granulocytes, permitted further studies to be undertaken into parallels between the processes differentiation in *T. annulata* and differentiation in higher eukaryotic cells. Commitment to granulocytic differentiation was found to be associated with a reversible increase in the expression of the g160 kDa antigen. In addition, alterations in proliferation relative to growth, by culturing towards stationary phase or by the use of aphidicolin, increased the level of g160 expression and, therefore, progression towards granulocytic differentiation. A model was proposed in which alterations in the concentration of key regulators of gene expression relative to their DNA regulatory sequences was linked to the probability of differentiation.

General parallels between differentiation in *Theileria* and similar events in both other protozoan parasites and in higher eukaryotes have been discussed (see 1.8. and Chapter 4). One possibility is that these studies reflect the operation of a primitive mechanism of cellular differentiation which has been retained by lower and higher eukaryotes. Further

investigation at the molecular level into these parallels would be aided greatly by the isolation of genes encoding both target DNA sequences and their respective regulatory protein factors. In *Theileria* putative DNA sequences and DNA binding factors have been recently identified, but await characterisation. However, the lack of a suitable transfection system for *Theileria* may limit further investigation of the regulation of gene expression. A clearer comparison between the general processes of differentiation in protozoan and in higher eukaryotic systems might be undertaken using a different parasitic system, for example the *in vitro* inter-conversion of bradyzoites and tachyzoites in *T. gondii*, where the techniques of transfection, mutagenesis and gene knockouts by homologous recombination are well established (Donald and Roos, 1994; Donald and Roos, 1995; Donald and Roos, 1993; Kim, *et al.*, 1993; Sibley, *et al.*, 1994). Whatever system is used, delineation of the mechanisms involved in differentiation of apicomplexan parasites from one life cycle stage to the next will provide valuable information on these fundamental biological events.

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## **Appendix**

**Map of the 5' nucleotide sequence of  $\lambda$  Zap cDNA clone 4A1 showing the deduced amino acid sequence for the 3 forward reading frames**

GAATTCGGCACCAAGTGAAGTACATTTATTTTTTACACTTCCCCAATGCACAAGTTGTTTTCC  
1 -----+-----+-----+-----+-----+-----+  
CTTAAGCCGTGGTCTTCACTGTAAATAAAAAATGTGAAGGGGTACGTGTTCAACAAAAGG

1 E F G T R S D I Y F Y T S P M H K L F S  
2 N S A P E V T F I F T L P Q C T S C F P  
3 I R H Q K \* H L F L H F P N A Q V V F P

CTTAAATAAAGGAGACACTTCACACCCCCAGACCTGGGATCTCAGATTTGTTCCCAACAT  
61 -----+-----+-----+-----+-----+-----+  
GAATTTATTTCTCTGTGAAGTGTGGGGTCTGGACCCTAGAGTCTAAACAAGGGTTGTA

1 L K \* R R H F T P P D L G S Q I C S Q H  
2 L N K G D T S H P Q T W D L R F V P N I  
3 \* I K E T L H T P R P G I S D L F P T F

TTCCATTCAAGTTGTGTATCCAGAAAGATGTCCAAAAGCAATGTTCTCTCTGCGGCATGT  
121 -----+-----+-----+-----+-----+-----+  
AAGGTAAGTTCAACACATAGGTCTTTCTACAGGTTTTCGTTACAAGAGAGACGCCGTACA

1 F H S S C V S R K M S K S N V L S A A C  
2 S I Q V V Y P E R C P K A M F S L R H V  
3 P F K L C I Q K D V Q K Q C S L C G M F

TCCTTGTTTGTAGTTTCCAGTCAGCCAACTGTCTTCCACCCGGCTTCTCTTTCCTTGTGA  
181 -----+-----+-----+-----+-----+-----+  
AGGAACAAACTCAAAGGTCAAGTTCGGTTTGACAGAAGGTGGGCCGAAGAGAAAGGAACACT

1 S L F E F P V S Q T V F H P A S L S L \*  
2 P C L S F Q S A K L S S T R L L F P C D  
3 L V \* V S S Q P N C L P P G F S F L V T

CATTTTGACTCCCAAGTCTTTGTGTATCCACACTTGACTTCCTGGGTTTCAGAAATCTGT  
241 -----+-----+-----+-----+-----+-----+  
GTAAAACAGAGGGTTTCAGAAACACATAGGTGTGAAGTGAAGGACCCAAAGTCTTTAGACA

1 H F D S Q V F V Y P H L T S W V S E I C  
2 I L T P K S L C I H T \* L P G F Q K S V  
3 F \* L P S L C V S T L D F L G F R N L W

GGTCAGGCCTGCTGCCACTGCCCAGGGAAGTAGTTCCTGAAGGGTCAGTTTCAGGGTCAC  
301 -----+-----+-----+-----+-----+-----+  
CCAGTCCGGACGACGGTGACGGGTCCCTTCATCAAGGACTTCCCAGTCAAAGTCCCAGTG

1 G Q A C C H C P G K \* F L K G Q F Q G H  
2 V R P A A T A Q G S S S \* R V S F R V T  
3 S G L L P L P R E V V P E G S V S G S Q

AGAGAGCACATCTGACCAAAATAAGTGTGAGCTCAGCACAGCACTCGTGAGCTCAGCATG  
361 -----+-----+-----+-----+-----+-----+  
TCTCTCGTGTAGACTGGTTTTATTACACTCGAGTCGTGTCGTGAGCACTCGAGTCGTAC

1 R E H I \* P K \* V \* A Q H S T R E L S M  
2 E S T S D Q N K C E L S T A L V S S A C  
3 R A H L T K I S V S S A Q H S \* A Q H V

TCACTGATGAGTCAGTAAATCAATCCCCTCTGATTGTTGTCTTCTGCTTCTGCTGCTAAG  
 421 -----+-----+-----+-----+-----+-----+  
 AGTGACTACTCAGTCATTTAGTTAGGGGAGACTAACAACAGAAGACGAAGACGACGATTC  
  
 1 S L M S Q \* I N P L \* L L S S A S A A K  
 2 H \* \* V S K S I P S D C C L L L L L L R  
 3 T D E S V N Q S P L I V V F C F C C \* G  
  
 GGTCAGCTGGCCACCCTGNAAGTTNTTGNCCCTNTTGGCAACCCAAGCACTTGGTGTATTT  
 481 -----+-----+-----+-----+-----+-----+  
 CCAGTCGACCGGTGGGACNTTCAANAACNGGANAACCGTTGGGTTCGTGAACCACATAAA  
  
 1 G Q L A T L ? V ? ? L L A T Q A L G V F  
 2 V S W P P ? K ? L ? ? W Q P K H L V Y F  
 3 S A G H P ? S ? ? P ? G N P S T W C I S  
  
 CTNATCTNCAAGCCCTGGNAAGGGNGCTGTTTTGAATTTGTGTTAACATTTTTAACCAGN  
 541 -----+-----+-----+-----+-----+-----+  
 GANTAGANGTTCGGGACCNTTCCCNCGACAAACTTAAACACAATTGTAAAAATTGGTCN  
  
 1 L I ? K P W ? G ? C F E F V L T F L T ?  
 2 ? S ? S P G K G A V L N L C \* H F \* P ?  
 3 ? L Q A L ? R ? L F \* I C V N I F N Q ?

**Map of the 5' nucleotide sequence of  $\lambda$  Zap cDNA clone 4E1A showing the deduced amino acid sequence for the 3 forward reading frames**

GAATTCGGCACGAGGAGCGTTTTTTAGCTTCGGAAAACATAAAGGGCGTACATTCATGTAC  
1 -----+-----+-----+-----+-----+-----+-----+  
CTTAAGCCGTGCTCCTCGCAAAAATCGAAGCCTTTTGTATTTCCCGCATGTAAGTACATG

1 E F G T R S V F S F G K H K G R T F M Y  
2 N S A R G A F L A S E N I K G V H S C T  
3 I R H E E R F \* L R K T \* R A Y I H V R

GTCTACAACAACGACTCCAGCTATGTGAACTGGATAAGGTCGTTGCCCGAACCTTCCGGG  
61 -----+-----+-----+-----+-----+-----+-----+  
CAGATGTTGTTGCTGAGGTCGATACACTTGACCTATTCCAGCAACGGGCTTGAAGGCC

1 V Y N N D S S Y V N W I R S L P E P S G  
2 S T T T T P A M \* T G \* G R C P N L P G  
3 L Q Q R L Q L C E L D K V V A R T F R E

AAATTGCTCGAATTCCTCGATTTTGCAAAAAAATATGAAATGTGCAAAAGCCCAGAAAAT  
121 -----+-----+-----+-----+-----+-----+-----+  
TTTAACGAGCTTAAGGAGCTAAAACGTTTTTTTTTATACTTTACACGTTTTTCGGGTCTTTTA

1 K L L E F L D F A K K Y E M C K S P E N  
2 N C S N S S I L Q K N M K C A K A Q K I  
3 I A R I P R F C K K I \* N V Q K P R K \*

AATACTAAGGATTCCATGCAAATTCAACATAATTACCCCAAATGTGTCCAAACAAATGAT  
181 -----+-----+-----+-----+-----+-----+-----+  
TTATGATTCTTAAGGTACGTTTAAGTTGTATTAAGTGGGTTTACACAGGTTTGTTTACTA

1 N T K D S M Q I Q H N S P K C V Q T N D  
2 I L R I P C K F N I I H P N V S K Q M I  
3 Y \* G F H A N S T \* F T Q M C P N K \* S

CATTCAGGTCTTTTATCACCAAATGCCAATAGAGCTTCTCTAGAATCCAATTTTTTCTCT  
241 -----+-----+-----+-----+-----+-----+-----+  
GTAAGTCCAGAAAATAGTGGTTTACGGTTATCTCGAAGAGATCTTAGGTTAAAAAAGAGA

1 H S G L L S P N A N R A S L E S N F F S  
2 I Q V F Y H Q M P I E L L \* N P I F S L  
3 F R S F I T K C Q \* S F S R I Q F F L \*

AAAAATAAAGATATAACACCGCTTAAATCATATAATACGAATAGTAATTTACGAAATAAT  
301 -----+-----+-----+-----+-----+-----+-----+  
TTTTTATTTCTATATTGTGGCGAATTTAGTATATTATGCTTATCATTAATGCTTTATTA

1 K N K D I T P L K S Y N T N S N L R N N  
2 K I K I \* H R L N H I I R I V I Y E I I  
3 K \* R Y N T A \* I I \* Y E \* \* F T K \* F

TTCAATAATAATAGTGATGCTGTTGTTAATAATTTGAAAAATGAGTCTAATGATAATTTG  
361 -----+-----+-----+-----+-----+-----+-----+  
AAGTTATTATTATCACTACGACAACAATTATTAACCTTTTTACTCAGATTACTATTAAC

1 F N N N S D A V V N N L K N E S N D N L  
2 S I I I V M L L L I I \* K M S L M I I W  
3 Q \* \* \* \* C C C \* \* F E K \* V \* \* \* F G

GAAGAAAAAACTGGTGCAGAAATAAGTAAGAAGAGGAAAAATAGCTAATAATCCGACACCA  
 421 -----+-----+-----+-----+-----+-----+  
 CTTCTTTTTTGACCACGTCTTTATTCATTCTTCTCCTTTTATCGATTATTAGGCTGTGGT  
  
 1 E E K T G A E I S K K R K I A N N P T P  
 2 K K K L V Q K \* V R R G K \* L I I R H Q  
 3 R K N W C R N K \* E E E N S \* \* S D T R  
  
 GACGATGTGACAATTACTAGAGATAAGGATTTTGAGGTAGAAAGGCACTGTTATCACCT  
 481 -----+-----+-----+-----+-----+-----+  
 CTGCTACACTGTTAATGATCTCTATTTCCTAAACTCCATCTTTCCGTGACAATAGTGGGA  
  
 1 D D V T I T R D K D F E V E R H C Y H P  
 2 T M \* Q L L E I R I L R \* K G T V I T L  
 3 R C D N Y \* R \* G F \* G R K A L L S P S  
  
 CACTGAAACACATGCTCGATATTTTTACATCAGAACAAAAGAAAGTAATATGGNCTCTGA  
 541 -----+-----+-----+-----+-----+-----+  
 GTGACTTTGTGTACGAGCTATAAAAATGTAGTCTTGTTTTCTTTCATTATACCNGAGACT  
  
 1 H \* N T C S I F L H Q N K R K \* Y G L \*  
 2 T E T H A R Y F Y I R T K E S N M ? S D  
 3 L K H M L D I F T S E Q K K V I W ? L M  
  
 TGATTGTGATGGTGTTAATATGCTGATGGGAAGTAGAGATCGAGGGGTTTGTGTGTCATTG  
 601 -----+-----+-----+-----+-----+-----+  
 ACTAACACTACCACAATTATACGACTACCCTTCATCTCTAGCTCCCCAAACAACAGTAAC  
  
 1 \* L \* W C \* Y A D G K \* R S R G L L S L  
 2 D C D G V N M L M G S R D R G V C C H W  
 3 I V M V L I C \* W E V E I E G F V V I G

**Map of the 5' nucleotide sequence of λ Zap cDNA clone 2C1A  
showing the deduced amino acid sequence for the 3 forward frames**

GGAATTCGGCACGAGATACTCAATATGCCAGCAAATTTGGAAAACCTCAGCAGTGGCCACA  
1 -----+-----+-----+-----+-----+-----+  
CCTTAAGCCGTGCTCTATGAGTTATACGGTCGTTTAAACCTTTTGAGTCGTCACCGGTGT

1 G I R H E I L N M P A N L E N S A V A T  
2 E F G T R Y S I C Q Q I W K T Q Q W P Q  
3 N S A R D T Q Y A S K F G K L S S G H R

GGACGGGAAAAGGTCAGTTTTCTTCCAATCCCAAAGAAAGGCACTGCCAAAGAACGCTC  
61 -----+-----+-----+-----+-----+-----+  
CCTGCCCTTTTCCAGTCAAAAGGAAGGTTAGGGTTTCTTTCCGTGACGGTTTCTTGCGAG

1 G R E K V S F P S N P K E R H C Q R T L  
2 D G K R S V F L P I P K K G T A K E R S  
3 T G K G Q F S F Q S Q R K A L P K N A Q

AAACTACCGCACAATTGCACTCATCTCACATGCTAGTAAAGTAATGCTCAAAATTCTCCA  
121 -----+-----+-----+-----+-----+-----+  
TTTGATGGCGTGTTAACGTGAGTAGAGTGACGATCATTTTCATTACGAGTTTAAAGAGGT

1 K L P H N C T H L T C \* \* S N A Q N S P  
2 N Y R T I A L I S H A S K V M L K I L Q  
3 T T A Q L H S S H M L V K \* C S K F S K

AGCCAGGCTTCAGCAGTACGTGAACTGTGAACTCCAGATGTTCAAGCTGGTTTTAGAAA  
181 -----+-----+-----+-----+-----+-----+  
TCGGTCCGAAGTCGTCATGCACCTTGACACTTGAAGGTCTACAAGTTCGACCAAAATCTTT

1 S Q A S A V R E L \* T S R C S S W F \* K  
2 A R L Q Q Y V N C E L P D V Q A G F R K  
3 P G F S S T \* T V N F Q M F K L V L E K

AGGCAGAGGAACCAGAAACCAACTGCCAAAATCCGCTGGATCATCGAAAAAGCAAGAGA  
241 -----+-----+-----+-----+-----+-----+  
TCCGTCTCCTTGGTCTTTGGTTTTGACGGTTTTAGGCGACCTAGTAGCTTTTTTCGTTCTCT

1 R Q R N Q K P N C Q N P L D H R K S K R  
2 G R G T R N Q T A K I R W I I E K A R E  
3 A E E P E T K L P K S A G S S K K Q E S

GTTTCAGAAAAACATCTATTTCTGCTTTATTCACTATGCCAAAGACTTTGACTGTGTGGA  
301 -----+-----+-----+-----+-----+-----+  
CAAAGTCTTTTTGTAGATAAAGACGAAATAAGTGATACGGTTTCTGAAACTGACACACCT

1 V S E K H L F L L Y S L C Q R L \* L C G  
2 F Q K N I Y F C F I H Y A K D F D C V D  
3 F R K T S I S A L F T M P K T L T V W I

TCACAATACACTGTGGGAAAATCTGAAAGAGATGGGAATACCAGACCAKCTGACCTGCCT  
361 -----+-----+-----+-----+-----+-----+  
AGTGTTATGTGACACCCTTTTAGACTTTCTCTACCCTTATGGTCTGGTMGACTGGACGGA

1 S Q Y T V G K S E R D G N T R P ? D L P  
2 H N T L W E N L K E M G I P D ? L T C L  
3 T I H C G K I \* K R W E Y Q T ? \* P A S

CTTGAGAAATTTATATGCAGGTCAGGAAGGAACAGTTAGAACTGGACATGTAACAGCAGA  
 421 -----+-----+-----+-----+-----+-----+  
 GAACTCTTTAAATATACGTCCAGTCCTTCCTTGTCAATCTTGACCTGTACATTGTCGTCT  
  
 1 L E K F I C R S G R N S \* N W T C N S R  
 2 L R N L Y A G Q E G T V R T G H V T A D  
 3 \* E I Y M Q V R K E Q L E L D M \* Q Q T  
  
 CTGGTTCCAAATAGGAAAAGGNGTAAGTCACGGCTGTATATTGTCACCCTGTTTATTTAA  
 481 -----+-----+-----+-----+-----+-----+  
 GACCAAGGTTTATCCTTTTCCNCATTCAGTGCCGACATATAACAGTGGGACAAATAAATT  
  
 1 L V P N R K R ? K S R L Y I V T L F I \*  
 2 W F Q I G K G V S H G C I L S P C L F N  
 3 G S K \* E K ? \* V T A V Y C H P V Y L T  
  
 CTTATATGCAAAGTACATCGTGAGAAATGCTGGNCTGGNAGAGACACANGCTAGANTCAA  
 541 -----+-----+-----+-----+-----+-----+  
 GAATATACGTTTCATGTAGCACTCTTTACGACCNGACCNTCTCTGTGTNCGATCTNAGTT  
  
 1 L I C K V H R E K C W ? G R D T ? \* ? Q  
 2 L Y A K Y I V R N A G L ? E T ? A R ? K  
 3 Y M Q S T S \* E M L ? W ? R H ? L ? S ?



**Map of the 5' nucleotide sequence of  $\lambda$  Zap cDNA clone 1C1A  
showing the deduced amino acid sequence for the 3 forward frames**

GAATTCGGCACGAGGAATTANGTCCTCATAGCAACCAAATTTTACCTTCCAATNTCCAAT  
1 -----+-----+-----+-----+-----+-----+  
CTTAAGCCGTGCTCCTTAATNCAGGAGTATCGTTGGTTTTAAATGGAAGGTTANAGGTTA

1 E F G T R N ? V L I A T K F Y L P ? S N  
2 N S A R G I ? S S \* Q P N F T F Q ? P M  
3 I R H E E L ? P H S N Q I L P S N ? Q C

GTGGATTTTTAGAATNTTACCTNCTCATGTTGGAAGTTCTTNTAGTTTCTTTGACACTAT  
61 -----+-----+-----+-----+-----+-----+  
CACCTAAAATCTTANAATGGANGAGTACAACCTTCAAGAANATCAAAGAACTGTGATA

1 V D F \* N ? T ? S C W K F ? \* F L \* H Y  
2 W I F R ? L P ? H V G S S ? S F F D T I  
3 G F L E ? Y L L M L E V L ? V S L T L S

CGACACTNCAGAGCTTATTTTGGCTCATTCCTCCTTGTACTGTTCAAGGCAGGTTCCCCA  
121 -----+-----+-----+-----+-----+-----+  
GCTGTGANGTCTCGAATAAAACCGAGTAAGGAGGAACATGACAAGTTCCGTCCAAGGGGT

1 R H ? R A Y F G S F L L V L F K A G S P  
2 D T ? E L I L A H S S L Y C S R Q V P H  
3 T L Q S L F W L I P P C T V Q G R F P T

CAGAAATTATTACCCATACAAGCTGCTCAAGCATCTAGATAGAAAATCCTGCAGCCCACA  
181 -----+-----+-----+-----+-----+-----+  
GTCTTTAATAATGGGTATGTTGACGAGTTTCGTAGATCTATCTTTTAGGACGTCGGGTGT

1 Q K L L P I Q A A Q A S R \* K I L Q P T  
2 R N Y Y P Y K L L K H L D R K S C S P H  
3 E I I T H T S C S S I \* I E N P A A H T

CTTGGGAGATGCTGTCACTGTTNGCTTACAGGTAACCCTTCTAATGAAGTTCCCTCCTTT  
241 -----+-----+-----+-----+-----+-----+  
GAACCCTCTACGACAGTGACAANCGAATGTCCATTGGGAAGATTACTTCAAGGGAGGAAA

1 L G R C C H C ? L T G N P S N E V P S F  
2 L G D A V T V ? L Q V T L L M K F P P L  
3 W E M L S L ? A Y R \* P F \* \* S S L L \*

AATCTCCANTNCACAGTTAGACACCTGCACTCACTTACCAAGATTCTTACTCAANANGAA  
301 -----+-----+-----+-----+-----+-----+  
TTAGAGGTNANGTGTCAATCTGTGGACGTGAGTGAATGGTTC TAAGAATGAGTTNTNCTT

1 N L ? ? T V R H L H S L T K I L T Q ? E  
2 I S ? ? Q L D T C T H L P R F L L ? ? N  
3 S P ? H S \* T P A L T Y Q D S Y S ? ? T

CTNAANTAAACCTATCANCTTCTTTCTNGGCTCTNTCTCCTNATCTCTCCATTTTNNTC  
361 -----+-----+-----+-----+-----+-----+  
GANTTNATTTGGATAGTNGAAGAAAGGANCCGAGANAGAGGANTAGAGAGGTAAAANNAG

1 L ? \* T Y ? L L S ? A L S P ? L S I ? ?  
2 ? ? K P I ? F F P ? L ? L L I S P F ? S  
3 ? ? N L S ? S F L G S ? S ? S L H F ? Q

AATNCTNNTNTCAACTNTCANCNGTNCCTANACACANTTACNGTCTTCTCCTTCACTNCC  
421 -----+-----+-----+-----+-----+-----+  
TTANGANNANAGTTGANAGTNGNCANGGATNTGTGTNAATGNCAGAAGAGGAAGTGANGG

1 N ? ? ? N ? ? ? ? L ? T ? T V F S F T ?  
2 ? L ? S T ? ? ? ? ? T ? L ? S S P S L P  
3 ? ? ? Q L S ? V P ? H ? Y ? L L L H ? Q

AANTCCANNGNTTATNGCCTCTCAAGTTCATTNCTTCTCTTCCTTTTCTNCTTCCTTCTT  
481 -----+-----+-----+-----+-----+-----+  
TTNAGGTNNCNAATANCGGAGAGTTCAAGTAANGAAGAGAAGGAAAAGANGAAGGAAGAA

1 ? S ? ? Y ? L S S S ? L L F L F ? F L L  
2 ? P ? ? ? A S Q V H ? F S S F S ? S F F  
3 ? ? ? L ? P L K F I ? S L P F L L P S S

CCTNNCTCCTNCCCCCTAAACANCTCANNTTGGGCATNAATCCCANCANCCCCCTAACCTN  
541 -----+-----+-----+-----+-----+-----+  
GGANNGAGGANGGGGGATTGTNGAGTNNNACCCGTANTTAGGGTNGTNGGGGATTGGAN

1 P ? S ? P L N ? S ? W A ? I P ? ? P N L  
2 L ? P ? P \* T ? ? ? G H ? S ? ? P L T ?  
3 ? L L P P K ? L ? ? G ? N P ? ? P \* P ?

**Map of the 5' nucleotide sequence of  $\lambda$  Zap cDNA clone 1E1A  
showing the deduced amino acid sequence for the 3 forward frames**

AACTAGTGATCCCCCGGGCTGCAGGAATTCGGCACGACTCTCTTTAGTTCTTTGTACGGA  
1 -----+-----+-----+-----+-----+-----+  
TTGATCACTAGGGGGCCCCGACGTCCCTTAAGCCGTGCTGAGAGAAATCAAGAAACATGCCT

1 N \* \* S P G L Q E F G T T L F S S L Y G  
2 T S D P P G C R N S A R L S L V L C T E  
3 L V I P R A A G I R H D S L \* F F V R R

GGTGAATAAAAACAAAAAGATCCCTTCAAAAATGGCAATGTATCTGAATTCCTCAAGCAGG  
61 -----+-----+-----+-----+-----+-----+  
CCACTTATTTTTGTTTTTCTAGGAAGTTTTTACCGTTACATAGACTTAAGGAGTTCGTCC

1 G E \* K Q K D P S K M A M Y L N S S S R  
2 V N K N K K I L Q K W Q C I \* I P Q A G  
3 \* I K T K R S F K N G N V S E F L K Q A

CCTGTTTATTGAAAATTAAGACAGGAATACATTAAAACGGTGAAAGGATCCAAAGGTGGT  
121 -----+-----+-----+-----+-----+-----+  
GGACAAATAACTTTTAATTCTGTCCCTATGTAATTTGCCACTTTCCTAGGTTTCCACCA

1 P V Y \* K L R Q E Y I K T V K G S K G G  
2 L F I E N \* D R N T L K R \* K D P K V V  
3 C L L K I K T G I H \* N G E R I Q R W S

CCTGGTCACAGTGCCCCCTATCCTACGTTCAATCCATCCTCGGATTTGAGGCCTTCACAAA  
181 -----+-----+-----+-----+-----+-----+  
GGACCAGTGTACGGGGATAGGATGCAAGTTAGGTAGGAGCCTAAACTCCGGAAGTGTTT

1 P G H S A P I L R S I H P R I \* G L H K  
2 L V T V P L S Y V Q S I L G F E A F T K  
3 W S Q C P Y P T F N P S S D L R P S Q S

GCAATCACAGTTAAAGGTTGATTAAGCAACCATCATTTAAATTCTACTAAAAACAATCA  
241 -----+-----+-----+-----+-----+-----+  
CGTTAGTGTCAATTTCCAACATAATTCGTTGGTAGTAAATTTAAGATGATTTTTTTGTTAGT

1 A I T V K G \* L S N H H L N S T K K Q S  
2 Q S Q L K V D \* A T I I \* I L L K N N H  
3 N H S \* R L I K Q P S F K F Y \* K T I T

CACTCACRGGATCAAAGCGCCTATCTCAGGAGAAAAGAAACCCCCTGGTAAGTTCTAAGAA  
301 -----+-----+-----+-----+-----+-----+  
GTGAGTGYCCTAGTTTCGCGGATAGAGTCCTCTTTCTTTGGGGGACCATTCAAGATTCTT

1 H S ? D Q S A Y L R R K K P P G K F \* E  
2 T H ? I K A P I S G E R N P L V S S K K  
3 L T G S K R L S Q E K E T P W \* V L R K

AGCCCTCCTCTCACCTTAGAATTTTTTGCTCTTTGATAACTTCATCCCTTTTTTTTGCCAA  
361 -----+-----+-----+-----+-----+-----+  
420 TCGGGAGGAGAGTGGAATCTTAAAAAACGAGAAACTATTGAAGTAGGGAAAAAACGGTT

1 S P P L T L E F F A L \* \* L H P F F C Q  
2 A L L S P \* N F L L F D N F I P F F A K  
3 P S S H L R I F C S L I T S S L F L P S

